Association of Uveal Melanocyte Destruction in Melanoma-Bearing Swine with Large Granular Lymphocyte Cells

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Sinclair strain miniature swine spontaneously develop and regress malignant melanoma lesions, with uveitis and vitiligo occurring subsequent to tumor regression. Peripheral blood lymphocytes (PBL) of Sinclair swine undergoing tumor regression and melanocyte destruction demonstrated significant lytic activity against K562, porcine semiallogeneic uveal melanocytes, and melanoma cells in 4-h chromium release assays. The ability of porcine PBL to lyse these target cells appears to be an age-associated immune response, as evidenced by the relative inability of PBL of pigs less than 4 weeks old to lyse target cells. In young adult pigs, however, PBL cytotoxic activity significantly increases; piglets 6 weeks old and older demonstrate efficient killing of all three targets. Conjugate formation assays demonstrate that a lymphoid effector cell possessing large granular lymphocyte (LGL) morphology may be involved in melanocyte destruction. These findings suggest that a LGL subpopulation may participate in melanoma and melanocyte destruction which can induce a uveitic syndrome in Sinclair swine with melanoma. Invest Ophthalmol Vis Sci 30:2455-2460, 1989

In Vogt-Koyanagi-Harada (VKH) disease, cytotoxic lymphocytes are postulated to mediate the destruction of melanocytes and thereby induce uveitis, vitiligo and other melanocyte-related pathology.1 The idea that lymphocytes function as cytotoxic effector cells in VKH disease is supported by the ability of peripheral blood lymphocytes (PBL) of VKH patients to kill allogeneic melanocytes and human melanoma cells.2-6 In addition, electron microscopy has shown that lymphocytes of VKH patients infiltrate and adhere to melanocytes of the uveal tract. Since normal melanocytes and malignant melanoma cells are derived from neural crest ectoderm, cytotoxic lymphocytes in VKH disease may recognize cell surface antigens common to both.7 A similar phenomenon apparently occurs in the Sinclair porcine model of cutaneous melanoma. Sinclair miniature swine spontaneously develop and regress malignant melanoma lesions,8 with uveitis and vitiligo occurring subsequent to tumor regression.9

Initial investigations of the Sinclair swine tumor regression–melanocyte destruction phenomenon suggested that immunological mechanisms may mediate these events.10 We examined the cytotoxic activity of Sinclair swine PBL against classic natural killer (NK)–sensitive target cell lines as well as against porcine uveal melanocyte and melanoma cell lines developed in our laboratory. In this report, we show that in Sinclair swine, a subset of cells within the LGL population display non–major histocompatibility complex (MHC)–restricted cytotoxicity against the three target cell lines. Thus, Sinclair swine may provide a unique animal model in which to examine the role of NK-like cells in melanocyte-associated diseases.

Materials and Methods

Animals

Sinclair miniature swine (S-1) originated from Hormel miniature swine and have been maintained at the University of Missouri as an outbred herd. The S-1 swine have a relatively low incidence (2%) of malignant melanoma. The Sinclair melanoma miniature swine were derived from the S-1 herd by repeated crossing of tumor-bearing S-1 stock. Sinclair melanoma swine are maintained as a line-bred herd with an 85% incidence of melanoma. The Sinclair melanoma swine were maintained under conditions rec-
ommended by the Guide for the Care and Use of Laboratory Animals (Bethesda, MD: National Institutes of Health, publication no. 86-23) in an American Association for Laboratory Animal Science (AALAS)-accredited facility, and investigations utilizing these animals conformed to the ARVO Resolution on the Use of Animals in Research.

Preparation of PBL Effectors

Serial, heparinized blood samples were collected from clinically healthy Sinclair melanoma swine by venipuncture of the cranial vena cava. Porcine peripheral blood lymphocytes were separated by diluting whole blood 1:2 with physiological buffered saline (PBS) and layering the cells onto a Ficoll-Paque density gradient (Pharmacia, Piscataway, NJ). The lymphocyte interface was collected, washed twice in PBS, resuspended and cultured overnight at 37°C in complete RPMI 1640 (Mediatech, Washington, DC) culture medium.

Preparation of Target Cells

K562 (American Type Culture Collection [ATCC], Rockville, MD), a human chronic myelogenous leukemia cell line, is known to be a NK-sensitive target. The K562 cell line was maintained under standard tissue culture conditions in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hazelton Research Products, Denver, PA), 200 mM L-glutamine (Mediatech, Washington, DC), and 40 mg/ml gentamicin (Elkins-Sim, Inc., Cherry Hill, NJ).

Porcine melanoma and porcine uveal melanocyte cell lines were derived using modified techniques previously described. Aseptically collected porcine tissue specimens were minced, collagenase-treated, resuspended, and cultured in supplemented Dulbecco's modified Eagle's medium (DME) (Mediatech, Washington, DC) media at 37°C in a 5% CO₂ chamber. After 48 hr of culture, uveal melanocytes were stimulated to proliferate with 10 ng/ml of phorbol myristate acetate (PMA) and 10 ng/ml of cholera toxin (CT). Both cell lines expanded rapidly and were maintained in complete media at 37°C in a 5% CO₂ chamber.

S 100 Staining

S 100 is a nervous-system-associated cytoplasmic protein which is also found in melanocytes and melanoma cells. The porcine melanocyte and melanoma target cell lines developed in our laboratory were stained with S 100 to verify the lack of fibroblast overgrowth. The neuroblastoma cell line, HTB-11 (ATCC, Rockville, MD), and K562 served as positive and negative controls, respectively. Porcine melanocytes and melanoma cells were allowed to attach to slides, fixed with cold methanol-acetone, and washed in PBS prior to staining. Rabbit anti-S 100 antibody (Biomeda Corp, Foster City, CA) was applied to the cells, which were then incubated in a humified 37°C chamber for 10 min. After incubation the slides were washed in PBS and goat anti-rabbit–conjugated fluorescein isothiocyanate (FITC) antibody was applied. Slides were then incubated for 20 min, in a 37°C humidified chamber. Slides were washed in PBS, coverslipped, and examined by fluorescent microscopy. Both melanocytes and melanoma cells were repeatedly shown to be positive for S 100 by this method.

Mycoplasma Testing

The Gen-Probe Mycoplasma Tissue Culture II Rapid Detection Kit (Fisher, St. Louis, MO) was used to evaluate the tissue culture cells for Mycoplasma contamination. The Gen-Probe Kit, a ribosomal RNA hybridization method, uses a DNA probe directed against the mycoplasma ribosomal RNA. Multiple testing with the Gen-Probe Kit determined the cell lines to be free of mycoplasma contamination.

Chromium Release Assays

Four-hour chromium (51Cr) release assays were performed using K562, porcine uveal melanocytes, and porcine melanoma cells as targets. K562 target cells were incubated for 4 hr with 100 µCi sodium chromate (51Cr) (New England Nuclear, Dupont, Boston, MA) on an orbital shaker at 37°C. Porcine uveal melanocyte and melanoma target cells were incubated for 6 hr with 100 µCi 51Cr on an orbital shaker at 37°C. After incubation, porcine uveal melanocytes and porcine melanoma cells were mechanically detached and washed in DME culture media. All three target cell lines were then washed twice in PBS, incubated at 37°C in 15 ml PBS for 30 min, washed, and resuspended at 1 x 10⁵ cells/ml. One hundred µl PBL effector cells (E) and 100 µl of 51Cr-labeled target cells (T) (1 x 10⁶) were added at a 50:1 effector:target ratio to wells of round-bottom 96-well microtiter plates. Target cells were incubated in media alone or with 1% NP40 to determine spontaneous and maximal release of chromium, respectively. All determinations were made in triplicate. After centrifugation at 1000 rpm for 3 min, the plates were incubated for 4 hr at 37°C, harvested with a Skatron supernatant collection system, and counted in a
gamma counter. The percentage of cytotoxicity was determined using the following formula:

$$\frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximal cpm} - \text{spontaneous cpm}} \times 100 = \% \text{ cytotoxicity}$$

Conjugate Assays

Uveal melanocytes or melanoma cells (1 $\times$ 10$^5$) were added to 6-well tissue culture plates and allowed to attach overnight. Porcine PBL (5 $\times$ 10$^6$) were added to wells containing either porcine uveal melanocytes or porcine melanoma cells. The porcine PBL were allowed to incubate with the target cells for 4 hr at 37°C. At the end of the 4-hr incubation period, nonadherent cells were gently aspirated from the wells, and the remaining adherent cell populations were stained with Wrights-Giemsa. Stained adherent cells were examined by light microscopy.

Flow Cytometry Analysis

Porcine PBL were incubated in 5% porcine plasma on ice for 20 min, washed, and resuspended in staining media (PBS + 0.1% bovine serum albumin + 0.1% sodium azide). Cells (1 $\times$ 10$^6$) were incubated on ice for 45 min with the appropriate antibody, washed, and stained with the appropriate fluorescein isothiocyanate (FITC)-labeled antibody. After incubating for an additional 45 min on ice, cells were washed and resuspended in staining media for flow cytometry analysis.14 The porcine monoclonal antibodies used in this study were MSA-4 (CD2)15 a gift from Dr. Craig Hammerberg (Virginia Polytechnic Institute, Blacksburg, VA), and monoclonal antibodies 74-12-4 (CD4) and 76-2-11 (CD8),15 gifts from Dr. David Sachs (National Cancer Institute, The National Institutes of Health, Bethesda, MD). The HNK-1 (TIB-200) antibody, which recognizes an antigen on human NK cells, was obtained from ATCC.

Statistical Evaluation

All cytotoxicity data derived from the two age groups were compared in pairs on each of the three target cell lines.16 The majority of data collected were longitudinal. However, due to experimental limitations, for a minority of animals only cross-sectional data were available. Therefore, statistical analysis of target cell lysis between groups consisted of conducting separate tests for matched and independent data. The matched data were compared using Wilcoxon's signed rank test; the independent data were compared using Wilcoxon's rank sum test. The $P$ values from these tests were weighted relative to the sample size and combined to generate the final results. A significance level of 0.05 was used.

Results

PBL collected from Sinclair melanoma swine of various ages were tested in 4-hr $^{51}$Cr release assays against K562 and semiallogeneic porcine uveal melanocyte and melanoma target cells. PBL from 2–4-week-old piglets displayed minimal cytotoxic activity against the NK-sensitive cell, K562 (Fig. 1). Similar to the immature Sinclair swine, mature domestic swine PBL demonstrated negligible lytic activity toward the target cell types tested (data not shown). In contrast, mature Sinclair swine PBL actively killed not only the NK-sensitive target cell line, K562 (Fig. 1), but also effectively lysed both porcine uveal melanocytes (Fig. 2) and melanoma cells (Fig. 3), as compared to 2–4-week-old pigs. The statistical difference for comparing different ages for each target cell line is reflected by the $P$-values: $P$-value K562
Fig. 3. Cytotoxicity of PBL against porcine melanoma cell line. 2–4-week-old pigs: 28 pigs/group; x = 1.2 ± 1.6. 6–10-week-old pigs: 16 pigs/group; x = 14.0 ± 7.0. Both age groups: P = 0.0010. x = the mean cytotoxicity ± the standard error of the mean. Each point represents one pig.

= .0014, P-value uveal melanocytes = .0011, and P-value melanoma = .0010.

As the Sinclair swine mature, gross tumor regression with subsequent signs of early depigmentation occurs. The tumor regression–depigmentation phenomenon correlated with a detectable increase in the ability of the porcine PBL to lyse chromium-labeled target cells. The ability to lyse a conventional NK-sensitive target cell as well as semiallogeneic porcine uveal melanocyte and melanoma targets indicates that a non–MHC-restricted lymphocyte population may be acting as an effector cell.

In addition to the chromium release assays, we added PBL from pigs displaying cytotoxic activity to either uveal melanocyte or melanoma monolayers. A small percentage of PBL were attached to the monolayers. The majority of lymphocytes isolated by a Ficoll-Paque density gradient did not form conjugates and were removed by aspiration. The small number of PBL that were observed in conjugate formation with the target cells possessed LGL morphology (Fig. 4).

The lymphocyte population containing the LGL effector cells capable of forming conjugates with the target cells that were separated on Ficoll-Paque gradients were subjected to phenotypic analysis by flow microfluorimetry. The phenotyping results demonstrated that approximately 10% of the lymphocytes expressed the markers detected by the HNK-1 antibody (Fig. 5).

Discussion

VKH syndrome is clinically manifested by vitiligo, intraocular inflammation, chorioretinitis, and patchy depigmentation of the fundus. The association of vitiligo with ocular abnormalities has been well documented. Dermal and uveal melanocytes, which originate from neuroectoderm, are morphologically similar. The vitiligo observed in melanoma patients correlates with an immune-mediated destruction of dermal and uveal melanocytes.

The PBL of VKH patients are capable of lysing allogeneic melanocytes and melanoma cells. Mazawa and Yano have shown that lymphocytes expressing the CD4 and CD8 markers in the PBL of VKH patients recognize P-36 melanoma cells. Sinclair swine with tumors develop a generalized vitiligo,
chorioretinitis, and iridial and fundus depigmentation. In this report we show that the PBL of pigs less than 4 weeks old demonstrate little, if any, lytic activity against K562, melanoma, or melanocyte target cells. Our findings are in general agreement with Kim, who reported that NK cells develop 2 weeks and 3–4 weeks postnatally in specific pathogen-free and germ-free piglets, respectively. However, exposure of these pigs to anaerobic microbial flora resulted in significant NK activity within the first 2 weeks of life. In Sinclair swine, melanoma lesions do not appear to accelerate the maturation or differentiation of NK activity since the swine display large progressively growing tumors and normal (black) pigment during the first 4 weeks of life. However, as the pigs mature, the onset of enhanced PBL cytotoxic activity against K562 cells, uveal melanocytes, and melanoma cells closely parallels the gross observation of tumor regression, generalized vitiligo, chorioretinitis, and iridial and fundus depigmentation. Sinclair swine PBL appear to mediate target cell lysis in a non-MHC-restricted manner, as evidenced by their ability to kill the NK-sensitive K562 cells in addition to the semiallogeneic porcine melanoma cells and uveal melanocytes. Non–MHC-restricted target cell lysis suggests that these porcine effector cells are either NK-like cells or non–MHC-restricted T cells.

In addition to circulating lymphocyte effector cells, cytotoxic lymphocytes infiltrating the uveal tract of VKH patients have been demonstrated. Recently, we isolated uvea-infiltrating lymphocytes (UIL) from a primary uveal melanocyte culture derived from a 6-week-old Sinclair swine displaying early signs of depigmentation. These lymphocytes were observed to adhere and lyse uveal melanocytes. These cells were phenotyped by flow cytometry and were determined to be $\text{CD}^+$, $\text{CD}^-$, $\text{CD}^-$, and HNK-1$^+$. This
phenotype suggests that an NK-like cell may be involved in uveal melanocyte destruction. We are currently examining the uvea-infiltrating lymphocytes for functional activity.

In summary, we show that Sinclair melanoma swine display an age-dependent cellular cytotoxicity against melanoma cells and melanocytes. In vitro melanocyte cytotoxicity data suggests that cell-mediated melanocyte destruction may be associated with generalized depigmentation and the development of uveitis in Sinclair swine. Although the maximal percent lysis generated by porcine PBL was only 30%, this percent lysis is significant in that chromium release assays were performed with an unenriched E subpopulation comprising only 10% of the total lymphocyte population. The lymphoid E subpopulation appears to be a NK-like cell based on the observation of non-MHC-restricted lysis of K562 and melanoma and melanocyte target cells, expression of HNK-1 marker, and LGL morphology. Morphologically, LGL appear to be a uniform population; however, they are heterogeneous both functionally and phenotypically. LGL fractions may contain NK cells, ADCC killer cells, and T cells that are lytic toward K562 and express shared markers (eg, CD2, CD3, HNK-1). Increasing evidence suggests that NK or ADCC cells may play an important role in the pathogenesis of many diseases, including VKH.

Key words: uveal melanocytes, melanoma, uveitis, LGL

References
22. Minato N, Takashi A, Yodoi J, Diamanstein T, and Kano S: T lymphoid E subpopulation comprising only 10% of the total lymphocyte population. The lymphoid E subpopulation appears to be a NK-like cell based on the observation of non-MHC-restricted lysis of K562 and melanoma and melanocyte target cells, expression of HNK-1 marker, and LGL morphology. Morphologically, LGL appear to be a uniform population; however, they are heterogeneous both functionally and phenotypically. LGL fractions may contain NK cells, ADCC killer cells, and T cells that are lytic toward K562 and express shared markers (eg, CD2, CD3, HNK-1). Increasing evidence suggests that NK or ADCC cells may play an important role in the pathogenesis of many diseases, including VKH.