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Effects of autologous stromal cells and cytokines on differentiation of equine bone marrow–derived progenitor cells

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Abstract

OBJECTIVE—To develop an in vitro system for differentiation of equine B cells from bone marrow hematopoietic progenitor cells on the basis of protocols for other species.

SAMPLE—Bone marrow aspirates aseptically obtained from 12 research horses.

PROCEDURES—Equine bone marrow CD34⁺ cells were sorted by use of magnetic beads and cultured in medium supplemented with cytokines (recombinant human interleukin-7, equine interleukin-7, stem cell factor, and Fms-like tyrosine kinase-3), murine OP9 stromal cell preconditioned medium, and equine fetal bone marrow mesenchymal stromal cell preconditioned medium. Cells in culture were characterized by use of flow cytometry, immunocytofluorescence microscopy, and quantitative reverse-transcriptase PCR assay.

RESULTS—For these culture conditions, bone marrow–derived equine CD34⁺ cells differentiated into CD19⁺IgM⁺ B cells that expressed the signature transcription factors early B-cell factor and transcription factor 3. These conditions also supported the concomitant development of autologous stromal cells, and their presence was supportive of B-cell development.

CONCLUSIONS AND CLINICAL RELEVANCE—Equine B cells were generated from bone marrow aspirates by use of supportive culture conditions. In vitro generation of equine autologous B cells should be of use in studies on regulation of cell differentiation and therapeutic transplantation.

Development of B lymphocytes starts in the BM as differentiation of hematopoietic stem cells into B-lineage precursors and ultimately immature B cells that migrate to the periphery. The various developmental stages of B cells can be identified by the expression of CD antigens, concentrations of transcription factors (E2A, EBF1, and paired box 5), and rearrangement status of immunoglobulin H+L chains.¹ Although the transition of common lymphocyte progenitors into B-cell–restricted or –committed precursors is still poorly defined functionally, similar populations of early B cells, pro-B cells, and pre-B cells have been identified in mice and humans.¹ Currently, the consensus is that B-lineage–committed cells pass through a CD34⁺CD10⁺CD19[–] common lymphoid progenitor in the early B-cell

stage before they progress via CD34⁺CD10⁺CD19⁺ pro-B, CD34⁻CD19⁺ large pre-B I and II, and CD34⁻CD19⁺ small pre-B II into CD34⁻CD19⁺IgM⁺ IM-B cells.^{2,3}

Successful in vitro production of B cells can occur when hematopoietic stem cells are cocultured with BM stromal cells and soluble growth factors.⁴⁻⁸ These stromal cells provide essential cytokines and other factors that support hematopoiesis and express adhesion molecules that define niches similar to in vivo conditions.⁹⁻¹³ Although microenvironmental cues that direct early B-cell commitment and differentiation are not entirely understood, the capacity of stromal cell culture systems to support the development of B-lineage cells from hematopoietic precursor cells has been partially characterized and has contributed to the discovery of IL-7 as a key cytokine for the stromal-dependent phase of B-cell development in mice.¹⁴⁻¹⁹ Additional cytokines have been identified, including FLT3L, which has been found to markedly enhance B-cell lymphopoiesis in murine embryonic stromal cell coculture systems.²⁰⁻²²

Primitive nonlineage-restricted progenitors require the presence of stromal cells to support lymphopoiesis. Among stromal cell lines used in murine and human B-cell differentiation in vitro are OP9 cells (op9/op9 mice deficient for myeloid colony-stimulating factor) and cells of a murine marrow stromal cell line (ie, MS-5), which have been found to provide known and also undefined soluble proteins and cell-bound ligands that support lineage differentiation.^{14,19} Serum-free, stromal-free B-cell differentiation culture conditions require a combination of cytokines (IL-7, SCF, and FLT3L) to support lymphoid progenitor differentiation.¹⁹

Information on human B-cell differentiation lags behind information on mouse B-cell differentiation.^{4,5,23-26} For instance, the most effective methods for human B-cell lymphopoiesis involve the use of murine stromal cell lines and cord blood-derived hematopoietic stem cells, which have been found to be more robust and less fastidious than BM-derived hematopoietic progenitor cells.^{4,8} Differentiation of B cells has also been reported for stromal cell-free cultures by use of a 2-step culture system or by adding supernatants from mesenchymal stromal cell cultures.^{8,27} In another feeder cell-free in vitro system, CD34⁺ cells from cord blood or BM were cultured on plates coated with intercellular adhesion molecule-1-Fc in the presence of human IL-7, SCF, and FLT3L and subsequently maintained in cytokine-free medium.²⁸

To the authors' knowledge, in vitro conditions for B-cell differentiation for the equine species have not been described, and such information is essential for evaluation of defective B-cell differentiation observed in immunodeficiencies and assessment of corresponding treatment approaches. The goal of the study reported here was to establish a culture system based on methods for human and murine in vitro hematopoiesis to differentiate equine B cells from BM-derived CD34⁺ cells. The hypothesis was that equine B cells can differentiate from BM precursor cells in vitro. In addition, autologous or allogeneic stromal cells that supported equine B-cell development in vitro were analyzed.

Materials and Methods

Sample

Bone marrow aspirates were collected from 12 healthy horses (6 Thoroughbreds and 6 warmbloods; females and geldings; age range, 3 to 19 years) immediately after the horses were euthanized by other research groups at Cornell University. Protocols were approved by the Institutional Animal Care and Use Committee of Cornell University. The BM aspirates were collected aseptically from the sternbrae of each horse by use of an 11-gauge BM biopsy needle.^a For each harvest, 30 to 60 mL of aspirate was collected into 60-mL syringes that contained 25,000 to 50,000 U of preservative-free heparin sodium.^b

Enrichment of CD34 cell precursors from equine BM aspirates

Aspirates were diluted with PBS solution^c containing 0.5% BSA^c (ratio, 1 part aspirate to 2 parts PBS solution–BSA), and neutrophils were removed by the addition of carbonyl iron,^c incubation at 37°C for 30 minutes, and exposure to a magnet.^{29–31} The cell suspension was carefully layered over density gradient medium (density, 1.077),^d which was then centrifuged^e at $700 \times g$; cells at the interface were harvested, washed in PBS solution–BSA, and incubated for 30 minutes with anti-canine CD34 antibody^f (dilution, 1:10).^{32,33} Cells were washed several times, resuspended in PBS solution–BSA with 2mM EDTA,^g and incubated with immunomagnetic microbead-conjugated rat anti-mouse IgG1.^h The CD34⁺ cells were obtained by passing the microbead-labeled cells through a cell separation columnⁱ placed in a magnetic field^h that allowed retention of the target cells. The cell solution enriched for CD34⁺ cells was recovered by manually flushing the cell separation columnⁱ with PBS solution–BSA with 2mM EDTA. Cells then were centrifuged at $300 \times g$, supernatant was removed, and the cells were resuspended in culture medium. The total number of cells recovered was determined by use of a hemacytometer.^j Cell viability was determined by exclusion of trypan blue stain^g; aliquots were used to measure the number of CD34⁺ cells by use of flow cytometry and immunocytofluorescence microscopy.

To measure enrichment (percentage of CD34⁺ cells) in the sorted solutions (nondifferentiated cells before culture), immunophenotyping was performed by use of flow cytometry and a monoclonal antibody that differed from the one used for cell sorting. Cells were pelleted in aliquots (5×10^4 cells/well) in 96-well V-bottom plates^f and treated with an immunoglobulin Fc-receptor blocking step (incubation with 10% normal goat serumⁱ in PBS solution for 20 minutes). Cells then were pelleted and resuspended in unconjugated primary antibody (anti-equine CD34) and incubated for 45 minutes at 4°C. Cells subsequently were washed with PBS solution, secondary goat anti-mouse IgG (H+L)-FITC^k antibody was

^aJamshidi bone marrow biopsy needle, BD Biosciences, Mountain View, Calif.

^bSagent Pharmaceuticals, Schaumburg, Ill.

^cSigma-Aldrich, St Louis, Mo.

^dFicoll-Paque Plus, GE Healthcare Life Sciences, Pittsburgh, Pa.

^eSorvall Legend XT, Thermo Fisher Scientific, Ashville, NC.

^fBD Biosciences, Mountain View, Calif.

^gGibco Life Technologies, Thermo Fisher Scientific, Grand Island, NY.

^hMiltenyi Biotec, Bergisch Gladbach, Germany.

ⁱLS column, Miltenyi Biotec, Bergisch Gladbach, Germany.

^jNeubauer hemacytometer, Hausser Scientific, Horsham, Pa.

^kJackson ImmunoResearch Laboratories, West Grove, Pa.

added, and cells were incubated for 45 minutes. Cells were washed, fixed with 2% paraformaldehyde^c in PBS solution, and analyzed immediately by use of a fluorescence-activated cell-sorting flow cytometer^l equipped with 488- μ m argon and 635- μ m red diode lasers and analysis software.^m At least 10,000 nongated events were collected. Negative control samples were tested by omitting the primary antibody and incubating the cells with only the secondary antibody. The mean $\pm$ SD number of cells positive for the CD34 marker was calculated.

Immunocytofluorescence microscopy was performed to further characterize sorted cells for the presence of CD34⁺ precursor cells (primitive lineage) and CD34⁺CD19⁺ precursor cells (already beginning to differentiate into the lymphoid lineage in the BM). Cell aliquots (5×10^3 cells/reaction field) were placed on adhesive slidesⁿ and incubated at 37°C for 60 minutes.³⁴ The slides were developed to permanently anchor live cells to 12 hydrophilic reaction fields on a glass surface without loss of antigenicity. In addition, the hydrophobic coating surrounding the reaction fields prevented the cell solution and reagents from mixing with other fields, which enabled testing of multiple markers on the same slide. For single-color labeling of cell surface markers, cells were fixed in 4% paraformaldehyde^c in PBS solution, washed in PBS solution, and blocked by incubation with 10% normal goat serum at room temperature (20°C) for 60 minutes, which was followed by incubation with primary antibody (anti-equine CD34) at 4°C overnight. Slides were washed several times with PBS solution; slides then were incubated with secondary goat anti-mouse IgG (H+L)-FITC^k at room temperature for 60 minutes and mounted in DAPI-containing mounting medium.^o For double-color labeling, cells were incubated first with anti-equine CD34 antibody, which was followed by incubation with goat anti-mouse IgG, F(ab')₂-FITC.^k Subsequently, the cells were incubated with anti-equine CD19 antibody,^p which was followed by an additional blocking step of incubation with the unconjugated Fab fragment^k (to prevent nonspecific binding of rhodamine-conjugated IgG) for 30 minutes, and finally incubation with goat anti-mouse IgG (H+L)-rhodamine.^q

B-cell differentiation in the presence of autologous mesenchymal stromal cells, preconditioned media, and cytokines

Culture conditions supported the development of equine B cells from BM-derived CD34⁺-enriched cell solutions. Autologous stromal mesenchymal cells from these cell solutions concomitantly developed in these culture conditions.

Equine BM-derived CD34⁺-enriched cell solution (2×10^5 cells/well) was cultured for 3 weeks in medium consisting of α -minimum essential medium^g supplemented with 10% fetal bovine serum^g plus 1 $\times$ antimicrobials and antimycotics,^g rhIL-7 (20 ng/mL of culture medium),^c recombinant equine IL-7 (10 μ L/mL of culture medium) produced in our

^lFACSCalibur, BD Biosciences, Mountain View, Calif.

^mCell Quest, BD Biosciences, Mountain View, Calif.

ⁿPaul Marienfeld GmbH & Co, Bad Mergentheim, Germany.

^oVectashield, VectorLabs, Burlingame, Calif.

^pProvided by Dr. Douglas F. Antczak, Baker Institute for Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY.

^qMolecular Probes, Thermo Fisher Scientific, Eugene, Ore.

laboratory, rhSCF (100 ng/mL of culture medium),^g rhFLT3L (50 ng/mL of culture medium),^g and preconditioned media from both OP9 cells and FMSCs (each at 10% of the final volume of the culture medium). All cultures were performed in a humidified incubator^t that contained 5% CO₂ in air at 37°C. Twice each week, half of the medium was removed (without disturbing the cells at the bottom of the well), and it was replaced with the same volume of fresh medium containing the respective cytokines and preconditioned medium at double the concentration; therefore, cell concentration was not disrupted, and fresh culture medium elements were provided weekly throughout the culture period. Morphological aspects were assessed daily by use of an inverted phase-contrast microscope^s with camera.^t After cells were incubated for 3 weeks, small nonadherent cells (presumably progenitor cells, differentiating B cells, and myeloid cells) were harvested, and the number of viable cells per well was determined by use of a hemacytometer and exclusion of trypan blue stain.

After cells were cultured for 3 weeks, aliquots of nonadherent cells were analyzed by use of immunocytofluorescence microscopy for the presence of differentiated B cells on the basis of expression of CD19 and IgM markers and the signature transcription factors E2A and EBF. In addition, the presence of CD34⁺ cells (nondifferentiated or less differentiated cells) was assessed. For single-color labeling, cells were processed as described previously and incubated with primary antibody (anti-equine CD34, anti-equine CD19, or anti-human IgM, respectively). For IgM immunostaining, a permeabilization step (incubation with 0.5% Triton X-100^c in PBS solution for 15 minutes) was included prior to the blocking step because IgM is expressed intracellularly and on the cell surface, depending on the stage of B-cell differentiation. Goat anti-mouse IgG (H+L)-rhodamine^q was used as a secondary antibody, and cells were prepared in DAPI-containing mounting medium. For double-color labeling of transcription factors and surface markers, cells were initially fixed in 4% paraformaldehyde^c in PBS solution for 8 minutes, washed in PBS solution, and blocked by incubation with 10% normal goat serum as described previously. Subsequently, primary antibody anti-equine CD19 was applied and incubated overnight. The next morning, cells were washed, which was followed by incubation with goat anti-mouse IgG (H+L)-rhodamine^q at room temperature for 60 minutes. Cells were washed and permeabilized by incubation with 0.5% Triton X-100 in PBS solution for 15 minutes, which was followed by a blocking step (incubation with 5% donkey serum^p or 5% normal goat serum^k for 30 minutes). Antibodies directed against E2A or EBF were applied and incubated for 60 minutes; slides were washed and then incubated with donkey anti-rabbit IgG (H+L)-FITC^k or goat anti-mouse IgG (H+L)-FITC^k, respectively. Slides were washed several times with PBS solution, and the slides then were mounted in DAPI-containing mounting medium, stored at 4°C, and analyzed microscopically within the subsequent 2 weeks. Control experiments in which one of the primary antibodies was omitted but the secondary antibodies were applied did not reveal cross-reactivity between the 2 detection systems (data not shown).

^tVWR, Radnor, Pa.

^sCKX41 microscope, Olympus, Waltham, Mass.

^tDP12 digital camera, Olympus, Waltham, Mass.

Image acquisition and analyses

Fields on the adhesive slides were examined by use of a microscope^u with blue, green, and red filter packs. Digital images were obtained for analyses by use of a cooled charged-couple device camera^v and a color camera for bright-field images.^w For each digital image, blue (DAPI) plus red (rhodamine) or blue plus green (FITC) images were obtained; images of the individual fluorophores were overlaid for phenotypic evaluation.^x Depending on the number of cells in an image, 3 to 10 microscopic fields were examined for each pair of fluorophores (ie, DAPI and rhodamine or DAPI and FITC) and consecutive images of a microscopic field by use of the filters for DAPI, FITC, and rhodamine. Occasionally, bright-field images were acquired and merged with individual fluorophore images. Depending on the marker tested, merged images were analyzed for surface (ring, patched, or capped), nuclear, and cytoplasmic staining patterns. Digital images were converted to a TIF format for processing and subsequently imported to an imaging processor^y to increase image resolution to 300 dots/in.

Image analyses^x consisted of identifying and counting the fluorescence-associated cells and measuring fluorescence intensity of cells. First, the background of each image was determined by creating random regions in the image and measuring the mean intensity of each image; this constant was then subtracted from the image by use of an arithmetic function. To quantify the number of DAPI-labeled nuclei in each image, an autofluorescence threshold function was applied for light objects, and the objects were counted by use of the integrated morphometry analysis function. Total count of a standard area represented the total number of nuclei (ie, number of cells). To count the cells with positive FITC or rhodamine signals, the region measurement feature was applied, and regions were created around each cell associated with a positive fluorescent signal. The threshold function was applied to the image, and region measurements (including the region label and integrated fluorescence intensity of each cell) were saved in a logarithmic file.^z Thus, the number of fluorescence-associated cells was determined.

On the basis of the DAPI counts, the percentage of cells for each marker or marker combination was calculated for each image, and the distribution of percentages was examined across the cell culture wells. To measure a possible correlation between expression of E2A and CD19 as well as expression of EBF and CD19, pooled data from all double-positive images were used to generate scatterplots for each pair, and a linear trend was fitted to the data points.

Cytologic evaluation of in vitro-generated equine B lymphocytes

Approximately 1×10^4 to 5×10^4 in vitro-generated nonadherent differentiating cells were centrifuged^{aa} onto glass slides ($30 \times g$ for 3 minutes) and subsequently air dried. Slides were

^uBX50 Upright Microscope, Olympus Corporation of Americas, Center Valley, Pa.

^vRetiga EXi, QImaging, Surrey, BC, Canada.

^wMoticam, Motic North America, Richmond, BC, Canada.

^xMetamorph acquisition and analysis software, Molecular Devices, Sunnyvale, Calif.

^yAdobe Photoshop, version CS2, Adobe Systems, San Jose, Calif.

^zMicrosoft Excel, Microsoft Corp, Redmond, Wash.

^{aa}Shandon cytospin 2, Thermo Fisher Scientific, Ashville, NC.

stained by use of a Romanowski staining kit,^{bb} and a differential cell count (total of 200 cells counted/slide) was performed with a light microscope.^s

Characterization of BM autologous stromal mesenchymal cells

Bone marrow stromal cells support hematopoiesis in vivo and in vitro. By use of the culture conditions described previously, autologous mesenchymal stromal cells developed on the bottom of the culture wells. These adherent cells were harvested by use of cell-dissociation solution^{cc} and separately expanded in 25-cm² culture flasks^a containing F12–Dulbecco modified Eagle medium^g supplemented with 10% fetal bovine serum^g plus 1X antimicrobials and antimycotics^g at 5% CO₂ and 37°C to generate a sufficient number of cells for further characterization.

Autologous stromal cells were labeled for flow cytometric immunophenotyping by use of the previously described protocol and antibodies that defined these cells as described in the literature, including the expected positive markers (CD29 and CD90) and expected negative markers (CD14 and CD34).³⁵

For immunocytofluorescence microscopy, cells (after expansion) were again detached from flasks by use of the cell-dissociation solution^{cc} and seeded (1×10^4 cells/chamber) in chambered slides^{dd} coated with human placental collagen type VI.^c Cells were cultured for 2 to 4 days, at which time they reached confluence; cells then were washed with PBS solution and fixed in 4% paraformaldehyde,^c which was followed by an immunoglobulin Fc-receptor blocking step (incubation with 10% normal goat serum)^k and subsequent incubation with primary antibodies anti-human CD29, anti-equine CD90, or anti-equine CD34. The FITC-conjugated^k or rhodamine-conjugated^l secondary antibodies were applied, and cells were washed and processed in DAPI-containing mounting medium.

Autologous stromal cells were harvested for gene expression analysis by scraping cells from the culture wells and placing them in 3 mL of culture medium. Cells were centrifuged^e ($300 \times g$ for 10 minutes) and then transferred to microcentrifuge conical tubes,^k resuspended in 1 mL of PBS solution, pelleted, and stored at –80°C. The RNA was isolated by use of a kit.^{ee} One RNA sample isolated from snap-frozen adult BM tissue was included as an assay positive control sample. A 1-step RT-PCR assay^{ff} was performed with 100 ng of RNA. Equine mRNA-specific oligonucleotide primers were designed to include exon-intron boundaries (5′–3′). The PCR assay conditions^{gg} were 40 cycles of annealing temperature in the range of 55° to 58°C. Amplification products were resolved by use of electrophoresis on 1% (wt/vol) agarose gels^c and stained with a green fluorescent nucleic acid dye.^{hh}

^{bb}Diff-Quick, Thermo Fisher Scientific, Pittsburgh, Pa.

^{cc}Accumax, Millipore, Temecula, Calif.

^{dd}Nunc Lab-Tek II, Thermo Fisher Scientific, Rochester, NY.

^{ee}Qiagen RNeasy mini kit with on-column DNase I treatment, Qiagen, Valencia, Calif.

^{ff}SuperScript III 1-step RT-PCR kit, Thermo Fisher Scientific, Rockford, Ill.

^{gg}CFX96 real-time PCR detection system, Bio Rad, Hercules, Calif.

^{hh}GelGreen, Phenix Research Products, Candler, NC.

Primary antibodies used for flow cytometry, immunocytofluorescence microscopy, and immunocytochemical analysis

Antibodies used for flow cytometry, immunocytofluorescence microscopy, and immunocytochemical analysis were developed for equine molecules or have cross-reactivity for equine molecules.^{30,31,33,36–40} These included anti-canine CD34 (clone 1H6),^a anti-equine CD19 (clone cz2.1),^p anti-human IgM (clone CM7)ⁱⁱ for cytoplasmic and surface IgM, anti-human E2A (V-18),^{jj} anti-human EBF (C-8),^{jj} anti-human CD29 (4B4),^a anti-equine CD90 (DH24A),^{kk} and anti-equine CD14 (clone 105).^{ll} To measure the specificity and yield of the sorted BM cell precursors by use of the anti-canine CD34 monoclonal antibody, an anti-equine CD34 monoclonal antibody was developed by a company^{mm} and applied to the BM-sorted solutions.

Statistical analysis

Data distribution was normal as determined by use of the Shapiro-Wilk test and probability plot of residuals. A 1-way ANOVA with cell marker as the dependent variable and cell culture well as the independent variable was performed, followed by multiple pairwise comparisons with a Tukey-Kramer correction.ⁿⁿ Standard assumptions for a 1-way ANOVA were assessed for each model, and a value of $P < 0.05$ was used for all significance tests and confidence intervals.

To further characterize commitment regulation of B-cell lineage, correlation coefficients for expression of E2A and CD19 as well as expression of EBF and CD19 were calculated by use of the pooled integrated fluorescence intensities of all double-positive cells ($E2A^+CD19^+$ or EBF^+CD19^+) for all images obtained (harvested cells of various cultures or BM samples). Because the distribution for these data was not normal (Shapiro-Wilk test), square-root transformation of the data was performed to convert the data to a normal distribution. Finally, scatterplots were generated for each pair, and the correlation coefficient and linear regression lines were estimated.

Results

Phenotypic analysis of CD34⁺ cells and CD34⁺CD19⁺ cells from equine BM

A small aliquot of each BM-derived CD34⁺-enriched cell solution from various equine cadavers was processed for characterization before cell culture. Flow cytometric analysis revealed a high percentage of small cells with low granularity (compatible with BM cell precursors and lymphoid cells) in one region and a population of other cell lineages (eg, myeloid or stromal cells) in another region (Figure 1). Mean $\pm$ SD yield of CD34⁺ cells ($n = 4$ BM samples) in region 1 was $91.1 \pm 3.7\%$ of total cells in the gated area and $54.2 \pm 9.5\%$

ⁱⁱBioRad, Raleigh, NC.

^{jj}Santa Cruz Biotech, Dallas, Tex.

^{kk}Monoclonal Antibody Center, Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, Wash.

^{ll}Provided by Dr. Bettina Wagner, Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY.

^{mm}Biomatik, Wilmington, Del.

ⁿⁿJMP Pro, version 11, Cary, NC.

of total cells in the nongated area. Therefore, these solutions did not consist solely of CD34⁺ cells and contained other BM cells (including stromal cells) that codeveloped in culture.

Immunocytofluorescence microscopy of the sorted cells revealed polarized labeling of CD34⁺ cells, which suggested that cross-linking of the antigen by the antibody took place on the cell side facing the magnetic field (Figure 1). For quantitative assessment, images obtained for each experiment were analyzed by use of image morphometric analysis, and, on the basis of the total cell number, the percentage of CD34⁺ cells for each image was calculated. Double-stained immunofluorescence microscopy was used to quantitate the number of BM pro-B cells (CD34⁺CD19⁺) copurified with the CD34⁺ cells.

Overall, the mean $\pm$ SD percentage of sorted cells that were CD34⁺ cells was $67.8 \pm 13.2\%$ ($n = 60$ images for 6 BM samples; Figure 1). One BM sample had a significantly lower yield of CD34⁺ cells, compared with the number of CD34⁺ cells for samples with yields greater than the mean. Total number of CD34⁺ cells measured by use of flow cytometric analysis did not differ significantly ($P = 0.12$) from the value obtained by use of immunocytofluorescence microscopy.

Mean $\pm$ SD number of FITC-CD34⁺ cells that expressed the B-cell antigen rhodamine-CD19 and that had DAPI-stained nuclei was $7.1 \pm 5.3\%$ ($n = 52$ images for 6 BM samples; Figure 1). There were no significant ($P = 0.59$) differences among the BM samples. Few IgM⁺ cells were identified in freshly sorted CD34⁺ cells ($1.6 \pm 2.8\%$; 8 images for 3 BM samples).

Effects of autologous stromal cells plus a combination of preconditioned media and cytokines on equine B-cell differentiation

Autologous stromal cell development and B-cell differentiation were measured in equine BM-derived CD34⁺-enriched cell solutions cultured in preconditioned media (OP9 and FMSC) with cytokines (Figure 2). Within 2 weeks after initiation of cultures, the proportion of myeloid cells diminished and there was a progressive outgrowth of small round cells along the stromal cells. Lymphoid cells of small to medium size and several myeloid cells were seen in centrifuged preparations.

After cells had been cultured for 3 weeks, they were carefully harvested (stromal cells were left behind) and transferred to adhesive slides for further characterization by use of immunofluorescence microscopy. Most cells had CD19⁺ and IgM⁺ membrane staining (Figure 3). Because the cells were permeabilized prior to incubation with IgM antibody, a few had diffuse cytoplasmic staining. Only a few cells were CD34⁺ cells.

The percentage of CD19⁺ or IgM⁺ cells was calculated for each image on the basis of the total number of cells (ie, DAPI-stained nuclei). The percentage of CD19⁺ cells in the various cell culture wells ranged from 30.6% to 90% ($n = 86$ images of 10 cell culture wells for 5 BM samples; Figure 3). Most of the cultures had mean values close to the overall mean ($53.5 \pm 20.9\%$). Three cultures of cells from 1 BM sample had the highest percentage of CD19⁺ cells, whereas another culture originating from that same BM sample had the lowest percentage.

Mean $\pm$ SD percentage of IgM⁺ cells was $58.8 \pm 20.9\%$ ($n = 52$ images of 9 culture wells for 3 BM samples), which suggested that most of the CD34⁺ cells developed into B cells (Figure 3). Again, the yield of IgM⁺ cells differed significantly among cultures originating from the same BM sample.

Few CD34⁺ cells were measured in the cell cultures ($2.2 \pm 3.9\%$; $n = 27$ images of 8 culture wells for 3 BM samples; Figure 3). The number of CD34⁺ cells did not differ significantly ($P > 0.23$) among cultures.

Cells were processed in medium containing DAPI and stained for B-cell nuclear transcription factors E2A and EBF and the B-cell signature surface marker CD19 to further characterize the stage of B-cell differentiation (Figure 4). Quantitative analysis revealed that the mean $\pm$ SD percentage of E2A⁺CD19⁺ cells was $57.3 \pm 18.8\%$ ($n = 34$ images of 8 cultures for 4 BM samples) and the percentage of EBF⁺CD19⁺ cells was $51.8 \pm 24.0\%$ (26 images of 7 cultures for 4 BM samples). Several cultures from the same BM donor had significant differences in the yield of E2A⁺CD19⁺ and EBF⁺CD19⁺ cells. The mean percentage of E2A⁺CD19⁻ cells ($12.6 \pm 8.5\%$; range, 3.5% to 22.9%; 25 images of 7 culture wells for 4 BM samples) was greater than the percentage of CD34⁺ cells after 4 weeks of culture. No EBF⁺CD19⁻ cells were detected by use of immunofluorescence microscopy.

To measure a possible association between expression of E2A and CD19 as well as expression of EBF and CD19, pooled data from all double-positive images were used to generate scatterplots for each pair, and a linear trend was fitted to the data points. The correlation coefficient for expression of E2A and CD19 was 0.62 ($n = 854$ E2A⁺CD19⁺ cells of 9 culture wells for 4 BM samples) and for expression of EBF and CD19 was 0.72 (442 EBF⁺CD19⁺ cells of 6 culture wells for 3 BM samples; Figure 4).

B-cell differentiation in the absence of equine autologous BM stromal cells

Stromal cells occasionally did not develop in cell cultures (Figure 5). For these cultures, the overall mean $\pm$ SD percentage of CD19⁺ cells was $21.6 \pm 15.5\%$ ($n = 21$ images of 4 culture wells for 4 BM samples), which differed significantly ($P = 0.003$) from the overall mean percentage of CD34⁺ cells ($61.6 \pm 23.5\%$; 14 images of 4 culture wells for 4 BM samples). This reinforced the importance of the supportive role of stromal cells in B-cell differentiation. There were no significant differences among culture wells with regard to the percentage of CD19⁺ ($P > 0.29$) or CD34⁺ ($P > 0.053$) cells.

Characterization of BM autologous mesenchymal stromal cells

Autologous mesenchymal stromal cells that developed along with the CD34⁺-enriched cell culture had a characteristic elongated fibroblast-like morphology. A few stromal cells appeared, typically within 5 days after initiation of culture, and rapidly proliferated within 2 to 3 weeks to form a monolayer (Figure 6). Mesenchymal stromal cells were expanded for subsequent flow cytometry and immunofluorescence microscopy (chambered slide), and relatively pure stromal cell cultures were obtained for passages 1 through 3. Flow cytometric analyses of cells at passages 3 through 5 yielded positive results for the adhesion molecule CD29 (mean $\pm$ SD, $97.1 \pm 4.3\%$; $n = 5$) and the mesenchymal cell marker CD90 (mean, 97.9

$\pm 2.9\%$; 6) and negative results for CD14 (mean, $6.2 \pm 3.9\%$; 3) and CD34 (mean, $4.3 \pm 2.9\%$; 6).

Concurrently, cells were seeded in chambered slides for immunostaining. Consistent with the results for flow cytometry, most of the stromal cells were CD29⁺ and CD90⁺ cells, and few were CD34⁺ cells (Figure 6). It has been reported in previous studies^{41,42} that a lack of CD34 expression may be a consequence of culture conditions; therefore, stromal cells were harvested directly from the original cell culture well (approx 3 to 4 weeks later without further culture) and analyzed by use of flow cytometry. Again, only a few cells were positive for CD34.

Finally, autologous mesenchymal stromal cells were tested by use of RT-PCR assay for the production of cytokines supportive of B-cell lymphopoiesis. Stromal cells expressed IL-7, SCF, and FLT3L mRNA independent of the BM sample and passage number (Figure 6).

Discussion

The in vitro protocol used in the study reported here resulted in equine B-cell differentiation from BM-derived CD34⁺-enriched cell solutions. The B-cell differentiation required a combination of OP9 and FMSC preconditioned media, supplementation of equine and rh cytokines, and the presence of autologous BM-derived mesenchymal stromal cells that developed during culture. Currently, all ingredients for successful in vitro B-cell differentiation are not known; hence, the combination of supporting stromal cells for soluble and cell-surface bound molecules and supplementation with known essential cytokines appeared to provide the essential molecules that caused cell lineage differentiation. At the least, as determined on the basis of results of gene expression analysis (data not shown), the stromal cell preconditioned medium from OP9 cells plus equine FMSCs provided the essential murine and equine cytokines SCF, FLT3L, and IL-7 for in vitro B-cell development.^{4,7,8,43–45} The presence of autologous stromal cells was essential for B-cell differentiation, which suggested the need for critical soluble and cell-surface molecules expressed by these cells, their potential to inhibit development of myeloid cells, or a role for the creation of niches necessary for in vitro B-cell differentiation. Indeed, the percentage of CD34⁺ cells remained high and similar to the baseline seeding value (67.8%) in the absence of stromal cells, which indicated a lack of differentiation or a dormant state. Autologous stromal cells only developed primarily when preconditioned media from both OP9 cells and FMSCs were present in the medium but not in exclusive conditions (data not shown), which suggested that the supernatant of these cells provided essential complementary cytokines.

During the initial culture period, there were 3 observed phases of cell differentiation. During week 1, CD34⁺ seeding cells acquired a pole shape, with the formation of a leading edge at the front of the pole and a uropod at the rear of the pole, which was coincident with the appearance of a few fibroblast-like autologous mesenchymal stromal cells. During week 2, lymphoid differentiating cells formed small clusters that associated with small colonies of stromal cells. During week 3, colonies of stromal cells merged, and the differentiating cells expanded their populations on top of the stromal cell monolayer.⁴⁶ An outgrowth of myeloid

cells prior to the appearance of small round cells, as has been described in other studies,^{4,8} was not observed, but a few myeloid cells were detected after the 3-week culture period.

When sorting cells from BM by use of the CD34 antibody, there is a possibility for isolating cells already committed to lymphoid differentiation in addition to multipotent hematopoietic stem cells. The proportion of CD34⁺CD19⁺ pro-B cells detected by use of the sorting protocol in the present study was similar to the proportion described in a previous study⁴⁷ but not as high as the one reported in another study.²¹ The proportion of CD19⁺ and IgM⁺ B cells in the present study that differentiated in the culture system from sorted CD34⁺ cells (not lineage committed) versus already differentiating CD34⁺CD19⁺ pro-B cells present at day 0 was not completely defined. Nevertheless, on the basis of the observation that a mean of 68% of the sorted cells were CD34⁺ cells, and 7.1% of that population were CD34⁺CD19⁺ cells, we estimated that approximately 1×10^4 CD34⁺CD19⁺ cells were present on day 0 when 2×10^5 CD34⁺ cells were seeded in each culture well. This number was lower than the number of CD19⁺ cells harvested after culture for 3 weeks (mean, 4.9×10^4 cells/well; range, 2.4×10^4 cells/well to 7×10^4 cells/well), which suggested that B cells developed from primitive lymphoid progenitor cells. This observation was further supported by the fact that the yield of equine CD19⁺ cells in the culture system of the present study was similar to the number of developing B cells obtained in vitro from human BM-derived hematopoietic stem cells. Furthermore, our results were in agreement with results of another study⁵ in which freshly sorted CD19⁺ cells (including CD34⁺CD19⁺ pro-B cells) were cultured in preconditioned media plus a mixture of cytokines (without stromal cells) and the distribution of CD19⁺ cells decreased to 3.6%, which suggested that these cells did not expand their differentiated population. Finally, IgM⁺ cells were only detected after cells were cultured for 3 weeks, and no IgM⁺ cells were identified in the baseline CD34⁺-sorted cell population.^{5,8}

In vitro B-cell differentiation was further characterized on the basis of the expression of B-cell developmental transcription factors, and most of the cells after culture for 3 weeks were E2A⁺CD19⁺ cells (57.3%), with a few cells expressing high amounts of E2A and low amounts of CD19 or EBF⁺CD19⁺ (51.8%). A smaller population of cells was characterized as E2A⁺CD19⁻ cells (12.6%), and no EBF⁺CD19⁻ cells were detected. This latter finding may be explained by the fact that in multipotent progenitor cells, CD19 chromatin is first remodeled at the upstream enhancer after E2A binding, with subsequent EBF and paired box 5 expression in the progression from common lymphoid progenitor cells to pre-pro-B cells.⁴⁸ This pattern of transcription factor expression during B-cell differentiation also explains the stronger relationship detected between EBF and CD19, compared with the relationship between expression of E2A and CD19. Altogether, these data also supported the presence of cells in differentiation progression from less differentiated into B-cell lineage-committed cells.

Stromal cells support hematopoiesis in vivo and in vitro by secreting various molecules and creating niches for cell lineage differentiation and development.⁴² Investigators of 1 study⁶ found that the murine stromal cell line MS-5 supported differentiation of human progenitor B cells from cord blood-derived CD34⁺ cells, and the addition of rhSCF and rh granulocyte colony-stimulating factor enhanced the production of B cells. However, this effect was not

observed for the culture systems of the present study, perhaps because the equine CD34⁺ cells failed to recognize a potential membrane-bound ligand or ligands on murine MS-5 stromal cells because the opposite outcome was obtained with the autologous equine stromal cells. In addition, differences in cell stage (primitive vs lymphoid lineage-committed cells) may have major implications in the development of protocols for ex vivo hematopoietic cell expansion and differentiation.^{49,50}

Relevant functional and biological differences exist between human cord blood and BM progenitor cells in the production and yield of B cells in vitro.^{49,51–58} The number and generative capacity of human B-lymphocyte progenitors measured in vitro and in vivo are higher with umbilical cord blood than with adult or pediatric BM.⁵³ Cord blood-derived hematopoietic stem cells proliferate more rapidly in response to cytokine stimulation than do BM-derived cells.⁵² Hematopoietic stem cells from human cord blood, but not from adult BM, have the potential for IL-7-independent B-cell lymphopoiesis, and IL-7 is more critical in maintaining human B-cell production during adult life than during the neonatal period.⁵ Finally, investigators have described impaired B-cell lymphopoiesis in bovine adult cells versus fetal cells by use of an OP9 cell coculture system.⁵⁹ Consequently, only a few systems have been described that support the generation of mature B cells from adult BM-derived pro-B cells or CD34⁺ cells.^{7,28,45}

In the present study, results for an in vitro culture system that involved the use of adult equine BM-derived CD34⁺-enriched cells in the presence of OP9 and FMSC preconditioned media, equine and rh cytokines, and autologous mesenchymal stromal cells that developed during the 3-week incubation period were described. Use of the culture system will allow evaluation of normal and aberrant equine B-cell lymphopoiesis (eg, horses with common variable immunodeficiency) and the generation of autologous B-cell precursors and B cells for potential therapeutic transplantation.

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ABBREVIATIONS

BM	Bone marrow
BSA	Bovine serum albumin
DAPI	4'-diamidino-2-phenylindole
E2A	Transcription factor 3
EBF	Early B-cell factor
Fab	Antibody-binding fragment
Fc	Crystallizable fragment

FITC	Fluorescein isothiocyanate
FLT3L	Fms-like tyrosine kinase-3
FMSC	Fetal mesenchymal stromal cell
H+L	Heavy and light
IL	Interleukin
rh	Recombinant human
RT	Reverse transcriptase
SCF	Stem cell factor

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Figure 1.

Results for enrichment of CD34⁺ cells obtained after cell sorting of equine BM samples by use of magnetic beads. A—Representative flow cytometric side-scatter (SSC-H; cell granularity) versus forward-scatter (FSC-H; cell size) dot plot of samples before culture (n = 4 experiments) illustrates a high concentration of small, low-granularity cells in region 1 (R1) that is consistent with progenitor cells and lymphocytes, whereas region 2 (R2) comprises a population of larger cells that is consistent with myeloid cell lineages and stromal cells not eliminated during sorting. B—Photomicrograph of results for epifluorescence microscopy of sorted CD34⁺ cells attached to an adhesive slide, immunostained by use of an FITC-Fab-conjugated secondary antibody, and counterstained with DAPI (blue nuclei). Notice that most of the cells have polarized labeling for CD34 (green). Bar = 10 μ m. C—Photomicrograph of results to detect precursor cells that could have initiated B-cell differentiation in the host (ie, CD34⁺CD19⁺ cells). Adherent cells were blocked by incubation with an unconjugated-Fab fragment, labeled with anti-equine CD19 antibody, and stained with rhodamine-conjugated secondary antibody. There is a rare double-positive cell (arrowhead) with evenly distributed CD19 staining of the cell surface (red) overlapping the polarized staining for CD34 (which creates a yellow dot); most cells are negative for CD19 and have only the polarized staining for CD34 (green). Bar = 10 μ m. D and E—Graphs of the percentage of BM CD34⁺ (D) and CD34⁺CD19⁺ (E) cells after sorting (n = 6 experiments) for fluorescence microscopy. The DAPI-stained nuclei were counted by use of integrated morphometry analysis, and fluorescence-associated cells in the

rhodamine (CD19⁺) or FITC (CD34⁺) channels were counted with the region measurement function. Data are reported as mean diamonds, in which the group (experiment) mean is represented by the middle horizontal line within each diamond, and the 95% confidence interval is represented by the top and bottom points of each diamond; horizontal lines above and below the group mean within each diamond are called overlap marks, in which the group means are significantly different when the interval between these lines for one group does not overlap the interval between these lines for another group; in addition, the width of a diamond is proportional to the size of the group, and the black circles indicate the number of microscopic fields examined for each group. The overall mean for all samples is the horizontal line that crosses the entire graph. Analysis of the data indicated that equine BM cells before culture comprise mainly CD34⁺ cells, with a small population of CD34⁺CD19⁺ differentiating cells, and a few other noncharacterized cells. Notice that the scale on the y-axis differs between panels D and E. ^{a,b}Samples of BM with different lowercase letters differ significantly ($P < 0.05$; 1-way ANOVA followed by Tukey-Kramer correction).

Figure 2.

Photomicrographs of the development of lymphoid and stromal cells in cultures containing preconditioned media and cytokines. Equine BM-derived CD34⁺-enriched cell solutions were cultured in preconditioned media from OP9 cells and equine FMSCs supplemented with rhSCF, rhFLT3L, rhIL-7, and equine IL-7. A and B—Images of equine BM-derived CD34⁺-enriched cell solutions after culture for 5 days. Notice the pole shape, with formation of a leading edge at the front of the pole and a uropod at the rear of the pole. C through F—In this culture condition, autologous stromal cells developed into a monolayer on the bottom of the well and supported hematopoietic lineage differentiation. Colonies of small round differentiating hematopoietic cells on top of stromal cell monolayers are evident after culture for 3 weeks. G—Differentiation of lymphocytes (arrow) is evident, but myeloid cells (arrowhead) are also present in the cell culture supernatant. Romanowski stain; bar = 50 μ m in panels A through F and 10 μ m in panel G.

Figure 3.

Photomicrographs of differentiated lymphocytes cultured under the same conditions described in Figure 2. Cells were harvested, transferred to adhesive slides, and immunostained with primary antibodies against CD19 (A and B), IgM (C and D), and CD34 (E and F) followed by rhodamine-conjugated secondary antibody and DAPI counterstain, and graphs were created of the percentage of CD19⁺ (G), IgM⁺ (H), and CD34⁺ (I) cells. Photomicrographs were obtained in gray scale (A, C, and E) or in color (B, D, and F). Fluorescence images for each marker (red for rhodamine and blue for DAPI) were obtained and overlaid for phenotypic evaluation. In panels C and D, staining with antibodies against CD19 or IgM revealed a ring pattern (arrow) typical of cell membrane staining, and a few cells had diffuse cytoplasmic IgM staining when a permeabilization step was used. Bar = 10 µm. For quantitative analysis, consecutive images were obtained by use of the filters for DAPI and rhodamine; DAPI-stained nuclei were counted by use of integrated morphometry analysis, and fluorescence-associated cells in the rhodamine channel were counted by use of the region measurement function. Analysis of the data indicated that most of the nonadherent cells after culture for 3 weeks expressed the B-cell markers CD19 and IgM, and a small percentage were CD34⁺ cells. ^{a-c}Wells with different lowercase letters differ significantly ($P < 0.05$; 1-way ANOVA followed by Tukey-Kramer correction). *See* Figures 1 and 2 for remainder of key.

Figure 4.

Photomicrographs of differentiated lymphocytes cultured under the same conditions described in Figure 2 that were permeabilized and immunostained with primary antibodies against CD19 and transcription factors E2A and EBF followed by rhodamine-conjugated and FITC-conjugated secondary antibody and DAPI counter-stain (A through H); graphs of the percentage of CD19⁺E2A⁺ (I), CD19⁺EBF⁺ (J), and CD19⁺E2A⁺ (K) cells; and scatterplots of the correlation between CD19⁺ cells and E2A⁺ cells (L) and between CD19⁺ cells and EBF⁺ cells (M). Photomicrographs were obtained to detect blue for DAPI (A and

E), green for E2A (B), red for CD19 (C and G), overlap color for DAPI-E2A-CD19 (D), green for EBF (F), and overlap color for DAPI-EBF-CD19 (H); photomicrographs were obtained in gray scale (A through C and E through G) and in color (D and H). Bar = 10 μ m. For quantitative assessment of CD19⁺E2A⁺ and CD19⁺EBF⁺ cells, images obtained for each marker were analyzed. For panels L and M, fluorescence intensities measured for both pairs in all images obtained for cell culture wells and BM samples were pooled, and square-root transformation of the data was performed to convert the data to a normal distribution. The correlation coefficient and linear regression line of best fit were estimated. The combined expression of B-cell essential markers in a large number of cells supports the characterization of B-cell differentiation in vitro. ^{a-d}Wells with different lowercase letters differ significantly ($P < 0.05$; 1-way ANOVA followed by Tukey-Kramer correction). *See* Figures 1 and 3 for remainder of key.

Figure 5.

Graphs of the percentage of CD19⁺ cells (A) and CD34⁺ cells (B) for BM samples cultured in the absence of stromal cells. Cells were cultured for 3 weeks, and autologous BM stromal cells did not always expand in culture. Cells were harvested, transferred to adhesive slides, and immunostained with primary antibodies against CD19 (A) and CD34 (B) followed by FITC-conjugated secondary antibody and DAPI counterstain (not shown). Images of individual fluorophores were obtained for quantitative analysis; DAPI-stained nuclei were counted by use of integrated morphometry analysis, and fluorescence-associated cells were counted by use of the region measurement function. There were no significant differences among cell culture wells (1-way ANOVA followed by Turkey-Kramer correction; values were considered significant at $P < 0.05$). Analysis indicated that absence of stromal cells prevented B-cell differentiation, which suggests an essential role of stromal cells in lineage differentiation. *See* Figure 1 for remainder of key.

Figure 6.

Photomicrographs of autologous BM stromal cells (cultured under the same conditions described in Figure 2) after culture for 5 days (A), 12 days (B), and 3 weeks (C); flow cytometric side-scatter (SSC-H; cell granularity) versus mean fluorescence-intensity dot plots for equine stromal cells after immunostaining with the markers CD29 (D), CD90 (E), CD14 (F), and CD34 (G); photomicrographs of stromal cells harvested from cultures for characterization, transferred to chambered slides, and immunostained for the stromal cell markers CD29 (H; FITC-labeled cells are green), CD90 (I; FITC-labeled cells are green), and CD34 (J; rhodamine-labeled cells are red) followed by FITC-conjugated and rhodamine-conjugated secondary antibody, respectively, and DAPI counterstain; and a representative gel depicting results of mRNA expression detected by use of an RT-PCR

assay for RNA extracted from stromal cells of various passages for various separate BM cell cultures (K). In panel B, notice that cultured cells had acquired a fibroblast-like morphology and formed a monolayer by day 12. Bar = 50 μ m. The same is evident in panels H and I. Bar = 20 μ m. In panel K, lanes were as follows: 1 = RNA ladder, 2 and 5 = equine IL-7, 3 and 6 = equine SCF, and 4 and 7 = equine FLT3L for 2 equine BM stromal cell batches representing passage 2 (lanes 2 through 4) and passage 5 (lanes 5 through 7). Expected size of PCR products was as follows: equine IL-7 = 142 bp, equine SCF = 189 bp, and equine FLT3L = 142 bp. These results indicated that autologous BM cells have characteristics that support B-cell differentiation.