Erythropoietin Withdrawal Alters Interactions Between Young Red Blood Cells, Splenic Endothelial Cells, and Macrophages: An In Vitro Model of Neocytolysis

JoAnn Trial, Lawrence Rice, and Clarence P. Alfrey

ABSTRACT

Background: **We have described the rapid destruction of young red blood cells (neocytolysis) in astronauts adapting to microgravity, in polycythemic high altitude dwellers who descend to sea level, and in patients with kidney disorders. This destruction results from a decrease in erythropoietin (EPO) production. We hypothesized that such EPO withdrawal could trigger physiological changes in cells other than red cell precursors and possibly lead to the uptake and destruction of young red cells by altering endothelial cell–macrophage interactions, most likely occurring in the spleen.**

Methods: **We identified EPO receptors on human splenic endothelial cells (HSEC) and investigated the responses of these cells to EPO withdrawal.**

Results: **A monolayer of HSEC, unlike human endothelial cells from aorta, glomerulus, or umbilical vein, demonstrated an increase in permeability upon EPO withdrawal that was accompanied by unique morphological changes. When HSEC were cultured with monocyte-derived macrophages (but not when either cell type was cultured alone), EPO withdrawal induced an increased ingestion of young red cells by macrophages when compared with the constant presence or absence of EPO.**

Conclusions: **HSEC may represent a unique cell type that is able to respond to EPO withdrawal by increasing permeability and interacting with phagocytic macrophages, which leads to neocytolysis. (J Investig Med 2001;49:335–345) Key Words: erythropoietin • endothelium • macrophages • erythrocytes • spleen**

INTRODUCTION

Red-cell mass homeostasis and response to depletion are controlled by erythropoietin-stimulated bone marrow production.[1] By contrast, physiological decreases in red cell mass are observed only in response to the few circumstances in which an acute excess of red cells occurs. One circumstance is the shift of blood from the extremities to the trunk in astronauts ascending into microgravity, which leads to decreased plasma volume and decreased EPO secretion.[2–4] Another is the descent to sea level of individuals acclimated to high altitudes.[5–7] An artificial red-cell excess is created by autologous transfusions ("blood doping") in athletes seeking to enhance performance.[8] In all these conditions, subsequent rapid decreases in red cell mass occur by hemolysis in the next few days, followed by a decline in red cell production after 8 to 10 days. The rapid phase of red cell destruction consists of a preferential loss of the youngest circulating red cells, which we have termed neocytolysis.[9] Both the rapid destruction of young cells and the decline in production of new ones may depend on a drop in EPO levels.[9,10] How this fall may initiate neocytolysis is the subject of the current investigations.

It was previously believed that the action of EPO is highly specific to erythroid precursors,[11] but recent findings indicate that endothelial cells have EPO receptors and

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This work was supported by the National Space Biomedical Research Institute award NCC9-58, "Neocytolysis: Mechanisms and Limitations." Additional salary support was provided by the Houston Veterans Affairs Medical Center and NIH AI46285.

A portion of this work was presented at the 41st Annual Meeting of the American Society of Hematology, New Orleans, La, December 6, 1999.

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that they transmit signals in response to EPO.[12,13] The responses of these cells to EPO withdrawal have not been studied. Whereas exposure to EPO after growth in vitro without it induces a pro-angiogenic phenotype,[13] we propose that EPO withdrawal after such exposure may induce a different phenotype that includes neocytolysis, either directly by the endothelial cell, or more likely indirectly by stimulation of professional phagocytes. Erythrophagocytosis by splenic endothelial cells is seen in hemolytic anemias, but this is a minor aspect of erythrocyte destruction, and the bulk of erythrophagocytosis in vivo is carried out by macrophages that are adjacent to the endothelial cells.[14] We hypothesize that transmission of signals from the endothelial cell to the macrophage upon EPO withdrawal may lead to increased recognition of young red cells by the macrophage, perhaps via adhesion molecules that are differentially expressed on the youngest cohort of mature red cells.

METHODS

Reagents

Cacodylic acid and hexamethyldisilazane were purchased from Ted Pella (Redding, Calif). Thiocarbohydrazide and osmium tetroxide were purchased from Electron Microscopy Sciences (Ft. Washington, Pa). Tannic acid was purchased from Polysciences (Warrington, Pa). Silver nitrate, Triton X-100, and formaldehyde were purchased from Sigma Chemical (St. Louis, Mo).

Preparation of Magnetic Beads

Tosylactivated M-450 beads (Dynal, Lake Success, NY) were coated with the endothelial cell–specific lectin UEA-1 (*Ulex europaeus* lectin, Sigma) as previously described.[15]

Isolation and Culture of Endothelial Cells

Pieces of human normal spleen were obtained immediately after surgical removal in a patient with immune thrombocytopenia. Within 1 hour, the tissue was minced with scissors and then forced through a metal screen (Cellector 50 mesh screen, VWR, Houston, Tex) with the plunger from a disposable 20-mL plastic syringe to release cells from the fibrous tissue. The cells were washed twice with Hank's basic salt solution (HBSS, Gibco BRL, Grand Island, NY) and resuspended at 5×10^6/mL in RPMI-1640 (Gibco BRL) without supplements. UEA-1–coated beads were added to the cells at 50 μL/mL and the mixture rotated end-over-end for 30 minutes at 4°C. Endothelial cells that were bound to the beads were removed with a magnet and cultured in attachment factor-coated flasks in serum-free endothelial cell culture medium (CS-C serum-free medium kit, Cell Systems, Kirkland, Wash). Cells were frozen at each passage (serum-free freezing kit, Cell Systems) and were used for experiments at passages 3 through 6. At all passages 100% of the cells bound fluorescein-conjugated UEA-1 (Sigma), as well as antibodies against CDs 31, 34, 36 and von Willebrand's factor (Beckman Coulter, Miami, Fla) and vimentin (Dako, Denmark). Other sources of human endothelial cells (umbilical vein, glomerulus, and aorta) were obtained commercially (Cell Systems) and grown in the same way.

Demonstration of EPO Receptors

For surface immunofluorescence, cells were grown on collagen I–coated glass-slide chambers (BD BioCoat, Becton Dickinson & Co., Franklin Lakes, NJ) that was further coated with attachment factor. The cells were grown to subconfluence and then stained either with a monoclonal antibody (clone 38409.11, R&D Systems, Minneapolis, Minn) against the external domain of the human EPO receptor (EPO R) or with an affinity-purified goat antibody against human EPO receptors (R&D Systems) or control mouse or goat IgGs (R&D Systems). Reaction with the primary antibodies (1 μg) was performed at ambient temperature in complete medium for 40 minutes. This was followed by two washes with medium and the addition of 1 μg FITC-conjugated sheep affinity-purified $F(ab')_2$ anti-mouse Ig (ICN Pharmaceuticals, Costa Mesa, Calif) for the mouse monoclonal antibodies or 5 μg FITC-streptavidin (Zymed Laboratories, South San Francisco, Calif) for the biotinylated goat antibodies. After a 30-minute incubation at ambient temperature, the chambers were washed with medium twice and immediately analyzed on an Eclipse TE300 fluorescence inverted microscope (Nikon Instruments, Melville, NY). Images were captured by a color CCD camera and processed by GelExpert software (NucleoTech Corp., Hayward, Calif) to TIF files.

For immunoblotting experiments, cells were grown in the standard manner on attachment factor–coated flasks. For stimulation by EPO, 20 mU of human recombinant EPO (Epoietin Alfa Procrit, Ortho Biotech, Rattan, NJ) was added to cells. As a positive control for EPO R, K562 cells were grown in RPMI-1640 medium plus 10% fetal bovine serum (Hyclone, Logan, Utah). One confluent 25-cm^2 flask of EPO treated or untreated and 4×10^6 K562 cells were lysed in a detergent buffer and immunoprecipitated with a monoclonal antibody against EPO R (clone 38409.11, R&D Systems) or a nonbonding control Ig (R&D Systems). The immunoprecipitated proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and blotted to a nitrocellulose membrane. The blot was probed with an

affinity-purified goat anti-human EPO R (R&D Systems) followed by a horseradish peroxidase–conjugated antibody to goat IgG for 1 hour before development with 4-chloro-1-naphthol.

Effect of EPO Deprivation on the Permeability of a Confluent Endothelial Monolayer

Endothelial cells were cultured in inserts floored with a 0.4-μm pore-sized membrane (Transwell, Corning Costar, Cambridge, Mass) coated with type I rat-tail collagen (Becton Dickinson, Franklin Lakes, NJ). Cells were cultured for 4 days without EPO, 4 days with 20 mU EPO, or 3 days with EPO followed by 1 day without. All cultures were washed and given fresh medium on day 3, with the addition of fresh EPO to the second group only. Permeability was measured by the passage of fluorescein-inulin (10 μM, Sigma) from the upper chamber (the insert) to the lower chamber (the tissue culture well) within 1 hour. Samples from the lower chamber were read on a fluorescence spectrophotometer (Model 650–40, Perkin-Elmer, Norwalk, Conn). Cells in some wells were poisoned with 0.1% sodium azide at the end of the experiment to demonstrate maximal permeability.

Silver Staining of Endothelial Cells

Our method of silver staining was adapted from a previous report.[16] The endothelial cell monolayer was washed 3 times using HBSS with calcium and magnesium. Formaldehyde (3.7% in HBSS) was added for 5 minutes. The wells were washed twice with HBSS and then 0.25% silver nitrate in water was added for 1 minute. The wells were washed four times with HBSS and exposed to ultraviolet light for 5 minutes.

Isolation and Culture of Monocyte-Derived Macrophages

Blood from normal donors was anticoagulated with preservative-free heparin, layered over a Ficoll-Hypaque gradient (Histopaque-1077, Sigma) and the mononuclear cells collected. These were allowed to adhere in serum-free RPMI-1640 medium to plastic, 6-well plates (Nunclone, Fisher, Houston, Tex) that were coated with 20 μg/mL human plasma fibronectin (Sigma). After 1 hour, the nonadherent cells were removed and the remaining cells were cultured for 2 to 4 days in RPMI-1640 plus 10% fetal bovine serum. For removal from the plate, the adherent cells were washed on the plate with HBSS but without calcium and magnesium and scraped off the plastic using rigid Cell Lifters (Fisher, Houston, Tex).

Size Separation of Red Cells

Spherocytosis of red cells was induced by diluting heparin-anticoagulated blood 1:100 in 10X Dulbecco's PBS (DPBS, Gibco BRL) that had been diluted 1:20 with water. The cells were sorted for the largest and smallest 20% of cells based on their forward light scatter on a linear plot. Sorting was done on an Altra cell sorter (Beckman Coulter, Miami, Fla) at low pressure (8.9 psi, or 61.4×10^3 Pa) to avoid damage to the cells.

Neocytolysis Model In Vitro

Human plasma fibronectin (Sigma) at 20 μg/mL was added to 50% soluble collagen (Vitrogen 100, Collagen Corp, Palo Alto, Calif); 500 μL of the mixture was allowed to polymerize in each well of a 24-well plate (Corning-Costar, Acton, Mass). Macrophages from 2- to 3-day cultures were added at 3 to 500,000 per well and allowed to adhere to the collagen pad in serum-free RPMI-1640. Nonadherent cells were washed off the pads after 1 hour. HSEC at a ratio of one T-25 flask per each 24-well plate were added in CS-C medium. Cells were cultured for 4 days without EPO, 4 days with 20 mU EPO, or 3 days with EPO followed by 1 day without, with a medium change on day 3 as described above. Red cells sorted for small or large size were added at 10^6 per well and the plates were spun at 150*g* for 2 minutes. The plates were then placed at 37°C overnight in a humidified incubator supplied with 5% CO_2. Nonadherent red cells were removed by vigorously washing the wells 4 times with HBSS with calcium and magnesium. When no red cells could be seen above the pad by microscopy, the adherent cell layers were lysed by the addition of 125 μL of 2% Triton X-100. The lysate was removed from the wells after 30 minutes and the optical density of hemoglobin at 540 nm was determined by spectrophotometry.

Scanning Electron Microscopy Sample Preparation

Cells grown on glass coverslips or 50% hydrated collagen pads were rinsed three times with HBSS with calcium and magnesium and then placed in a fixative that contained 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 1 hour at ambient temperature. This and further processing were as previously reported.[17] The coverslips were washed with 0.1 M cacodylate buffer, pH 7.3, three times for 5 minutes. The samples were then fixed with 1% cacodylate buffered osmium tetroxide for 1 hour and washed with distilled water three times for 5 minutes. The samples were sequentially treated with filtered aqueous 1% thiocarbohydrazide for 10 min, aqueous 1% osmium tetroxide for 10 minutes, aqueous 1% tannic acid for 30 minutes, and aqueous 1% uranyl acetate overnight in the dark. The samples were

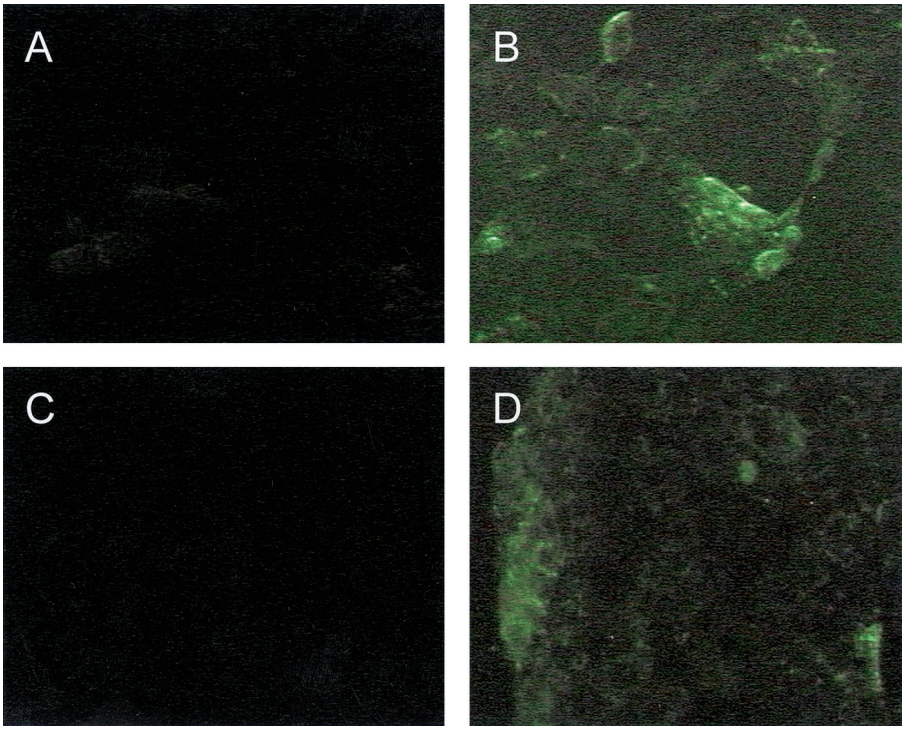

Figure 1. *Immunofluorescence of surface EPO receptors. HSEC were grown in glass-slide chambers until the cultures were subconfluent. The adherent cells were stained with control mouse IgG (A), a mouse monoclonal antibody against the external domain of the human EPO R (B), control goat IgG (C), or affinity-purified goat anti-EPO R (D).*

rinsed thoroughly with distilled water after every treatment to prevent any carryover of reagents. The samples were then dehydrated with a graded series of increasing concentrations of ethanol for 5 minutes each. The samples were then transferred to hexamethyldisilazane for 10 minutes and air dried overnight. Samples were mounted onto double-stick carbon tabs (Ted Pella, Inc.) that had been previously mounted onto aluminum specimen mounts (Electron Microscopy Sciences). The samples were coated under vacuum using a Balzer MED 010 evaporator (Hudson, NH) with platinum–palladium alloy for 2 minutes, then immediately carbon coated under vacuum for 2 minutes. The samples were transferred to a vacuum desiccator for examination at a later date. Samples were examined in a JSM-5900 scanning electron microscope (JEOL USA, Peabody, Mass) at an accelerating voltage of 5 kV.

Transmission Electron Microscopy Sample Preparation

Cells grown on glass coverslips were washed and fixed as described above for scanning electron microscopy. After fixation, the samples were washed and treated with 0.1% filtered cacodylate-buffered tannic acid, postfixed with 1% buffered osmium tetroxide for 1 hour, and stained en bloc with filtered 1% uranyl acetate. The samples were dehydrated with increasing concentrations of ethanol, infiltrated, and embedded in Spurr's low viscosity medium.

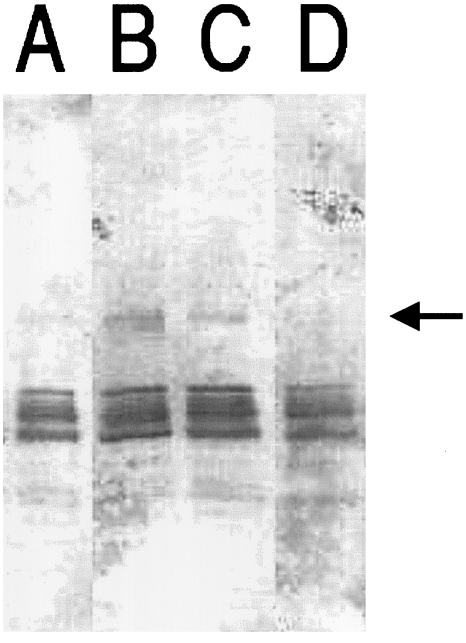

Figure 2. *Immunoblot of immunoprecipitated EPO receptors. HSEC were grown without EPO (lane A) or for 72 hours with 20 mU of EPO (lane B). K562 erythroleukemia cells were used as a control (lane C) to demonstrate valid EPO receptors. HSEC lysates were immunoprecipitated with a nonbinding mouse control Ig as a negative (lane D). The arrow indicates the position of the 78-kD EPO R.*

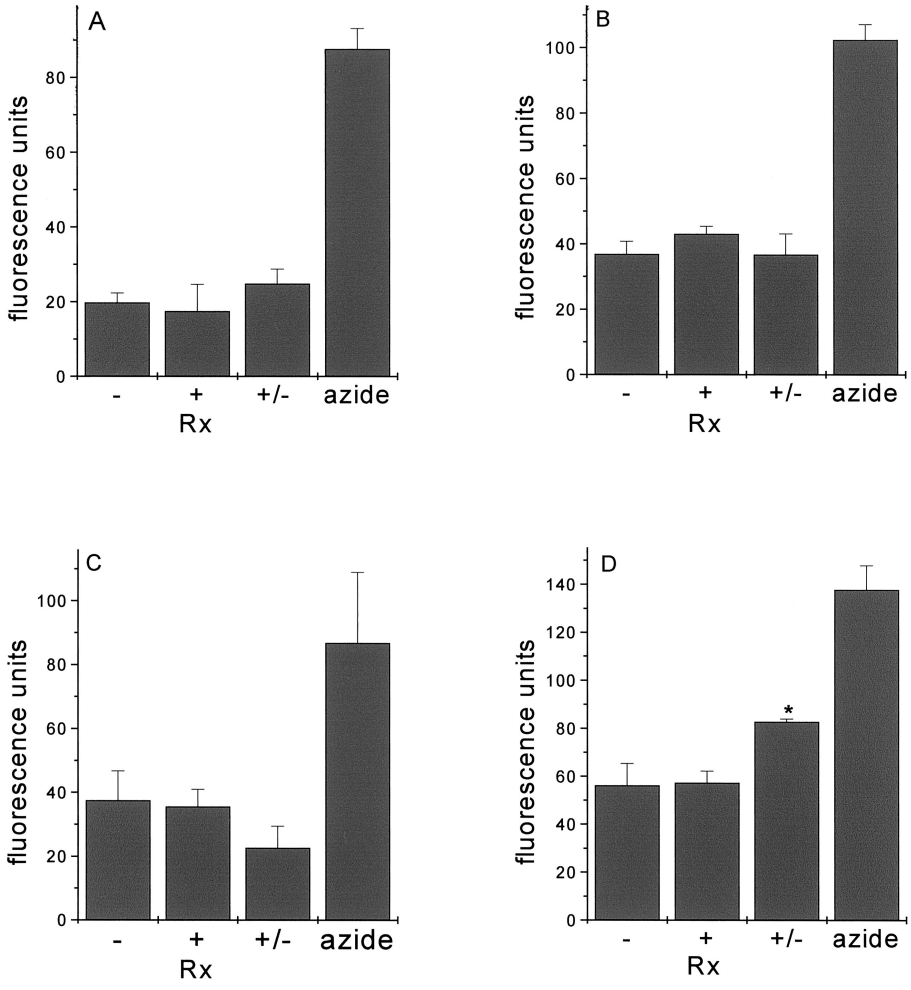

Figure 3. *Effect of EPO deprivation on the permeability of a confluent endothelial monolayer. Endothelial cells were cultured in inserts floored with a 0.4-μm pore-sized membrane and coated with collagen. Sources for endothelial cells were (A) human umbilical vein, (B) human glomeruli, (C) human aorta, and (D) human spleen. Confluency was confirmed by silver staining the monolayer. Cells were cultured for 4 days without EPO (−), 4 days with EPO (+), or 3 days with EPO followed by 1 day without (+/−). Permeability was measured by the passage of fluorescein-inulin from the upper chamber (the insert) to the lower chamber (the tissue culture well) within 1 hour. Samples from the lower chamber were read immediately on a fluorometer. Some wells were poisoned with 0.1% sodium azide at the end of the experiment to demonstrate maximal permeability. Data are pooled from four experiments performed in quadruplicate wells. The asterisk indicates statistical significance (P=0.04) of the (+/−) data compared to the (−) data (Student's* t *test).*

The samples were polymerized in a 60°C-oven for 2 days. The glass coverslips were removed by dipping the blocks in liquid nitrogen. Ultrathin sections were cut in an LKB Ultracut microtome, stained with uranyl acetate in an LKB Ultrostainer (Leica, Deerfield, Ill), and examined in a JEM 1010 transmission electron microscope (JEOL) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques, Danvers, Mass).

RESULTS

EPO Receptors on HSEC

Although EPO receptors have been reported on endothelium of other organs,[12,13] they have not been investigated on splenic endothelium. We used immunofluorescence to assess the presence of the receptors on HSEC. Whether we used a monoclonal antibody or affinity-purified goat antibodies against the EPO receptor, we saw immunofluorescence that was particularly noticeable at the edges of cell islands in subconfluent cultures (Figure 1). By contrast, almost no fluorescence was noted in confluent cultures (data not shown). To further investigate EPO receptors on these cells, we used the same antibodies to immunoprecipitate and immunoblot proteins from HSEC grown with and without EPO. We found a protein of the same molecular mass as the EPO receptor on K562 (Figure 2, lane C), a leukemic erythroid precursor, in HSEC grown in the presence of EPO but not without EPO (Figure 2, lane B vs lane A). Bands of a lower molecular weight than the EPO receptor were also seen in the sample of HSEC lysate that was prepared with a nonbinding mouse Ig (Figure 2, lane D) and in blots of purified protein A (data not shown), which indicates that these were contaminating proteins from the protein A beads used for the immunoprecipitation that may have bound the developing antibody or the enzyme substrate.

Responses to EPO Withdrawal

The finding of EPO receptors on HSEC prompted us to investigate whether these cells might be able to respond to EPO withdrawal, since this is thought to initiate neocytolysis in vivo. We chose to investigate permeability of the barrier formed by the confluent monolayer because this might relate to morphological changes that may lead to neocytolysis in vivo. HSEC and three other types of endothelium were grown to confluence in the presence of EPO, and then EPO was withdrawn. The ability of fluoresceinated inulin to pass through the endothelial monolayer during a period of 1 hour was investigated. Only HSEC allowed a greater amount of the fluorescent indicator to pass through the monolayer after EPO withdrawal (Figure 3).

Scanning electron microscopy of HSEC treated with and without EPO revealed flat cells with raised or folded borders, but it also revealed extensive development of microvilli and depressed borders after EPO withdrawal (Figure 4A–C). By contrast, human umbilical vein endothelial cells grown under these various conditions showed no distinguishing morphological differences based on treatment (Figure 4D–F). At harvest, all endothelial cells grown without EPO were confluent, as demonstrated by silver staining (Figure 5A). HSEC grown with EPO throughout and in the EPO-withdrawn cultures were confluent, although silver staining changed to a somewhat punctate pattern (Figure 5B). Transmission electron microscopy of the endothelial cells provided a possible explanation for the increased passage of the fluoresceinated inulin. EPO-withdrawn cells possessed large numbers of pinocytotic vesicles (Figure 6A) and possible vesiculo-vacuolar organelles (Figure 6B) not present in cells not exposed to EPO (Figure 6C). Further morphological changes observed were the presence of pits and dilated rough endoplasmic reticulum in EPO-withdrawn cells that were not seen in untreated cells. These features were not seen in any of 17 cells examined from untreated cells, which appeared morphologically quiescent. Pinocytotic vesicles were seen in all 20 of the treated cells examined, although possible vesiculo-vacuolar organelles were only seen in 5, perhaps because each section represents only a small portion of each cell.

To model neocytolysis in vitro, macrophages and young, as well as old, red cells were added to the HSEC culture system. The macrophages were obtained from normal donors by allowing monocytes to differentiate after adherence to fibronectin-coated plastic. We investigated the separation of old and young subpopulations of red cells by a variety of methods such as phthalate ester density

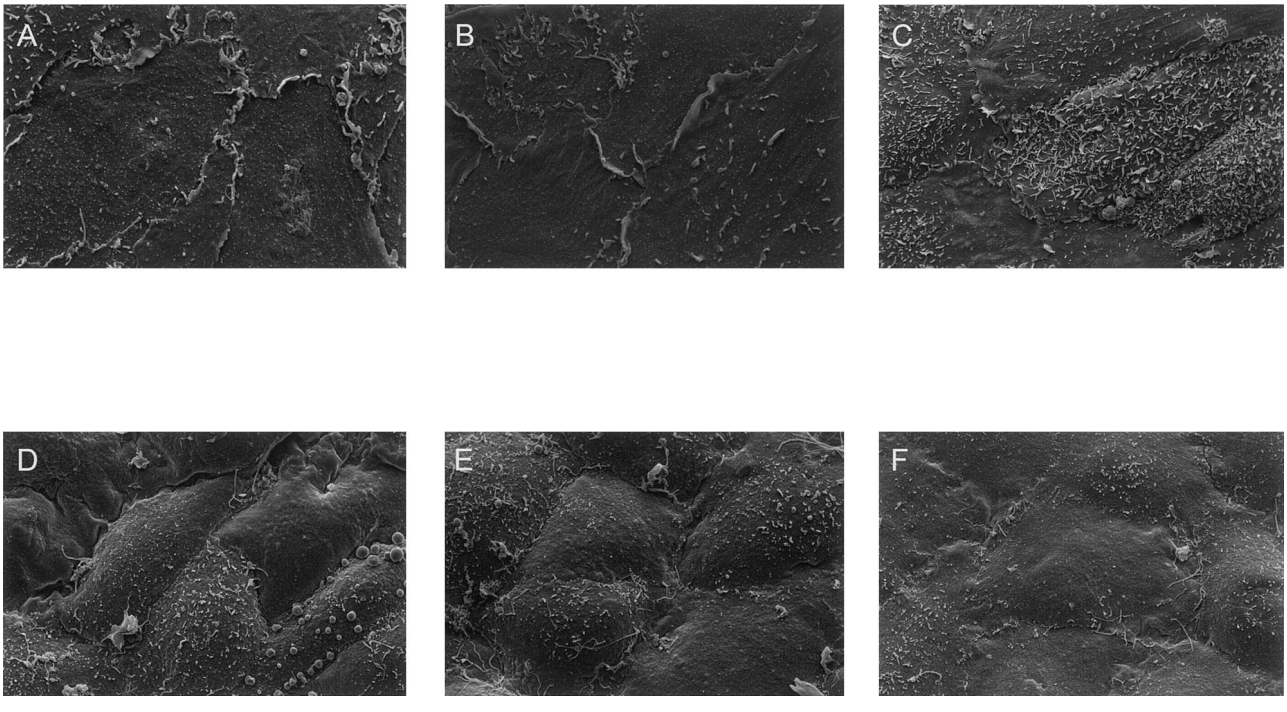

Figure 4. *Scanning electron microscopy of endothelial cells. Human splenic endothelium (A–C) and human umbilical vein endothelium (D–F) were grown on glass cover slips coated with attachment factor, without EPO (A and D), with EPO (B and E) throughout, or with EPO for 72 hours and without for the next 24 hours (C and F).*

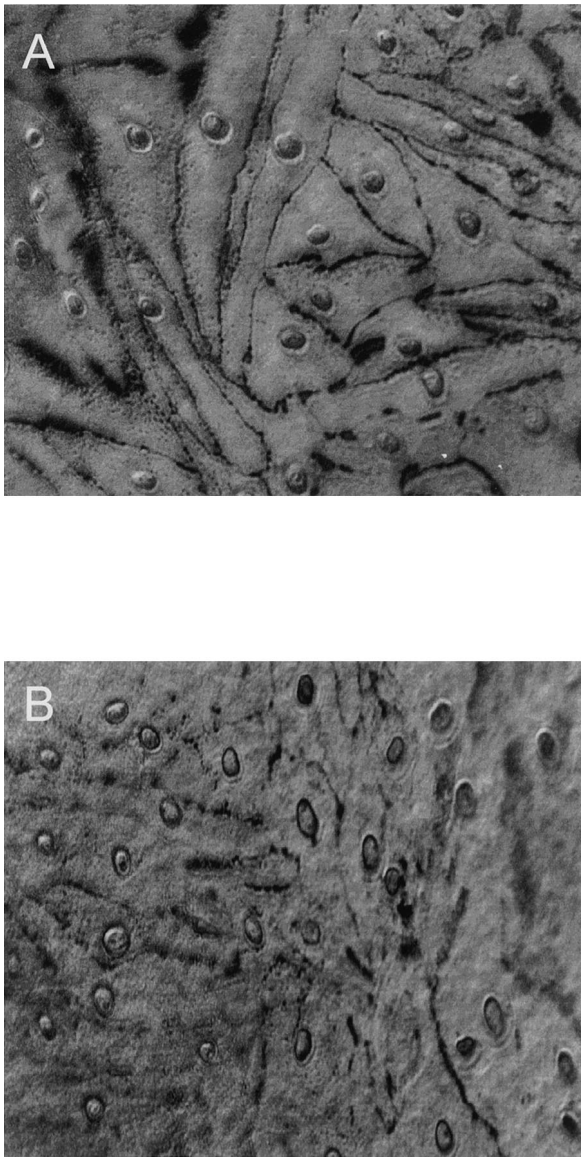

Figure 5. *Silver staining of HSEC. Cells on collagen pads grown without EPO (A) throughout or with EPO for 72 hours and without for 24 hours (B) were silver stained for confluency.*

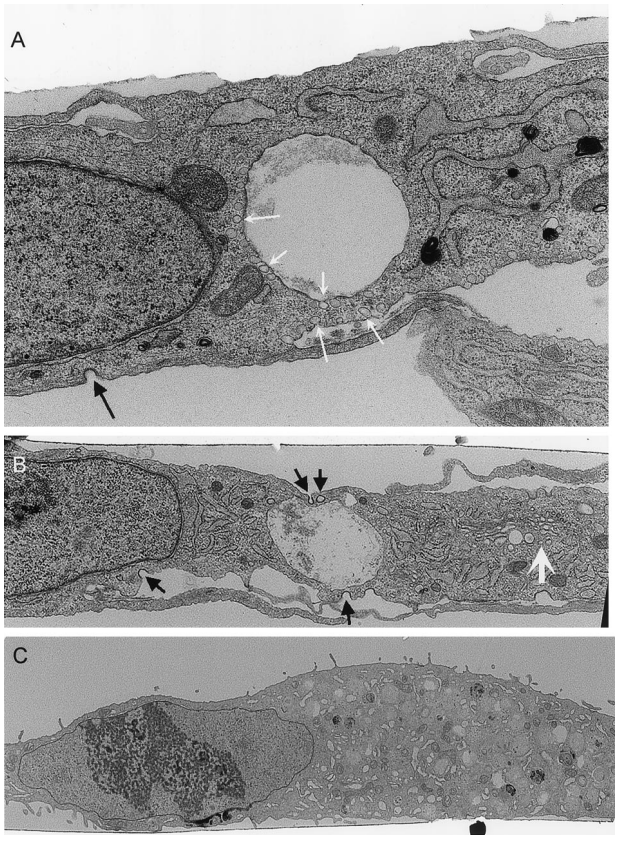

Figure 6. *Transmission electron microscopy of HSEC. HSEC grown on glass cover slips were EPO-withdrawn (A and B) or grown without EPO (C). Panel A shows an endothelial cell with pinocytic vesicles (white arrows) and a pit (black arrow). Panel B shows a cell with possible vesiculo-vacuolar organelles (white arrow) and pits (black arrows). Panel C shows a cell grown without EPO to demonstrate the normal absence of these organelles.*

gradients and sorting of cells based on their uptake of a fluorescent vital dye, but these altered the cell membranes such that macrophages ingested them indiscriminately. Since young red cells are larger than old red cells, we elected to sort two subpopulations from peripheral blood based on their size. To do this, we induced spherocytosis of the cells in a hypotonic buffer so that the orientation of the normally discoid cells would not yield spurious subpopulations and interfere with measurement of size based on forward-angle light scatter. The sorted populations maintained their size difference on reanalysis (Figure 7).

To assemble the cultures for the in vitro neocytolysis assay, monocyte-derived macrophages were scraped from their plastic cultures and allowed to adhere to pads formed of collagen plus fibronectin. Endothelial cells were plated on top of the adherent macrophages and treated according to a cycle of 72 hours with and 24 hours without EPO. Control groups were left untreated or treated with EPO throughout, with all groups receiving a medium change at 72 hours. Red cells were then sorted into young and old populations and added to the cultures. After an additional overnight culture, nonadherent red cells were removed by vigorous washing. All the remaining cells were lysed, and the hemoglobin content analyzed by spectrophotometry at 540 nm.

There was greater interaction of the adherent cells with old red cells as compared with young cells in all the cultures, except for the EPO-deprived cultures that cap-

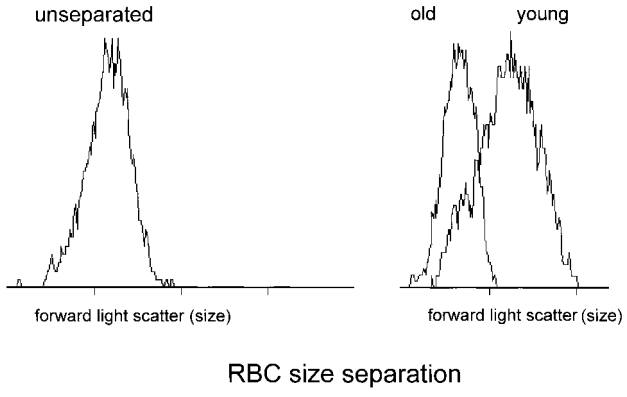

Figure 7. *Size separation of red blood cells (RBC). Red cells in fresh heparinized blood were made spherocytic by dilution into hypotonic PBS. Large versus small cells were sorted based on their forward light scatter. The left-hand graph describes the original unseparated population. The right-hand graph is an overlay of the sorted small (old) cells centered to the left and large (young) cells to the right.*

tured young red cells (Figure 8). This phenomenon was only seen after at least a 48-hour culture with EPO and its subsequent withdrawal (Figure 9). The capture required the presence of both the endothelial cells and macrophages, because cultures of either cell type alone treated in the same manner showed little interaction with and no preferential uptake of young red cells (Figure 10). Transmission electron microscopy of HSEC cocultured with macrophages demonstrated multiple layers of cells and their processes (Figure 11A). Scanning electron microscopy of EPO-withdrawn cocultures plus young red cells revealed exposure of macrophage processes above the endothelial cells; these processes bound red cells (Figure 11B). Transmission electron microscopy of such cultures showed macrophages with large phagosomes filled with membranes without subcellular organelles, which is consistent with red cells undergoing degradation (Figure 11C). Red cells could also be seen within macrophages by phase microscopy in cultures harvested at earlier time points than for electron microscopy (data not shown). Thus, upon EPO deprivation, signaling between the endothelium and adjacent macrophages may take place to allow uptake of young red cells by the macrophages.

DISCUSSION

The control of red cell numbers in the blood usually takes place by regulating red cell production in the bone marrow. When there is acute excess, however, red cell mass decreases too rapidly to be accounted for by a cessation of production. This decrease is owing to the preferential removal of the youngest mature red cells from

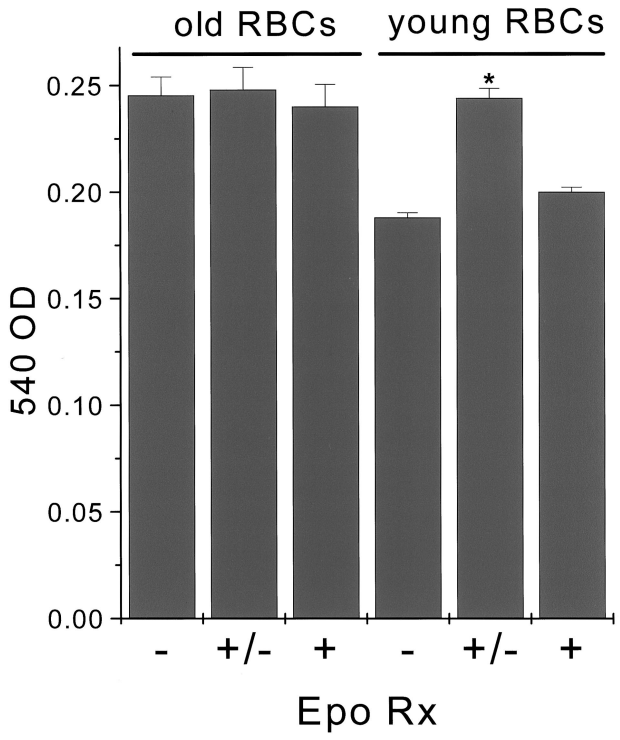

Figure 8. *Ingestion of young versus old red blood cells (RCBs) by cultures of HSEC and macrophages. Sorted red cells were given to composite cultures on collagen pads that had been grown without EPO (−), with EPO for 72 hours and without for 24 hours (+/−), or with EPO throughout (+). Data are pooled from five experiments performed in quadruplicate wells. The asterisk indicates statistical significance (P=0.04) of the (+/−) data compared with the (−) data (Student's* t *test). The y axis is the optical density at 540 nm, which was determined by spectrophotometry.*

the circulation (neocytolysis) in response to decreased levels of EPO, a process that is different from the normal turnover of aged erythrocytes.[9] We first discovered the physiological process of neocytolysis during our investigation of the anemia that invariably occurs in astronauts who return from even a few days in space.[4] On entering microgravity, the 20% of blood volume that is held in the extremities on earth suddenly pools centrally, leading to rapid transudation of plasma into tissues, hemoconcentration, acute plethora of red cells, and EPO suppression. Our studies indicate that it is EPO suppression well below its lowest physiological levels that initiates neocytolysis. This allows rapid adaptation of red cell mass in situations in which it would be excessive for a new environment. The existence of neocytolysis on earth without the contribution of space flight was confirmed when polycythemic residents of Cerro de Paseo, Peru (4380 m altitude), were transported to sea level, and low doses of EPO mitigated the rapid fall in red cell mass that normally occurs upon

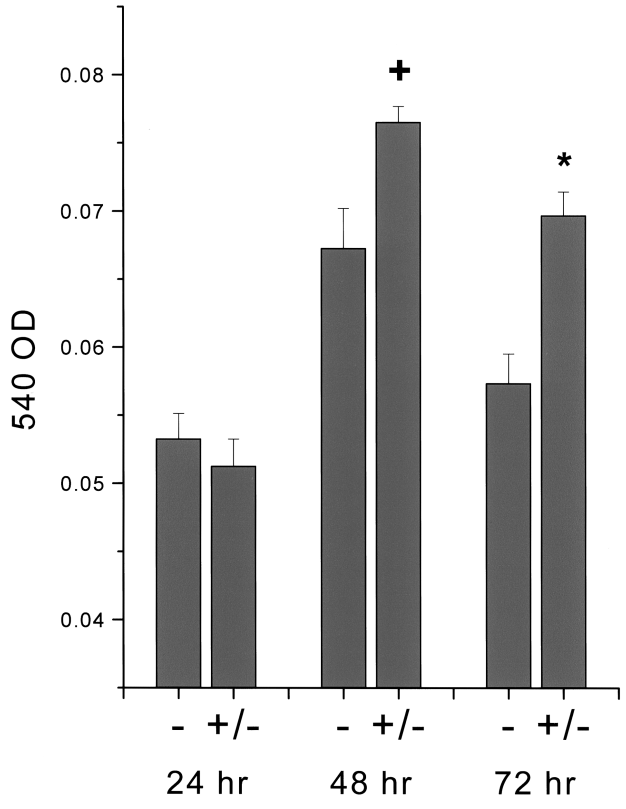

Figure 9. *Kinetics of response to EPO withdrawal. Composite cultures of HSEC on macrophages were cultured without EPO (−) or with EPO (+/−) for the indicated times, at which time the medium in all cultures was replaced and both groups were cultured without EPO for 24 hours. Sorted young red cells were given to all groups at the same time. Data are pooled from three experiments performed in sextuplicate wells. The plus sign indicates statistical significance* (P=*0.04) of the (+/−) data compared with the (−) data at 48 hours, and the asterisk indicates statistical significance of the (+/−) data compared with the (−) data at 72 hours (*P=*0.01). The y axis is the optical density at 540 nm, which was determined by spectrophotometry.*

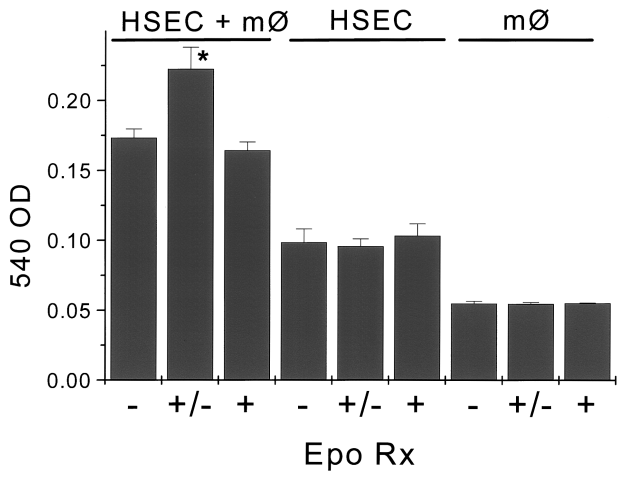

Figure 10. *Dependence on coculture for red blood cell ingestion. HSEC and macrophages (mØ) were cultured alone or together and their ingestion of sorted young red cells was measured. Cultures had been grown without EPO (−), with EPO for 72 hours and without for 24 hours (+/−), or uniformly with EPO (+). Data were pooled from two experiments performed in quadruplicate wells. The asterisk indicates statistical significance (*P=*0.05) of the (+/−) data compared with the (−) data (Student's* t *test). The y axis is the optical density at 540 nm, which was determined by spectrophotometry.*

this descent.[7] Neocytolysis also contributes to the anemia that occurs with pathological EPO deficiency and intermittent replacement therapy in renal disease.[18] We suspect that neocytolysis is involved in hemolytic disorders, in the adaptation of newborns to the post-uterine environment, and in the consequences of blood doping by endurance athletes. The data reported here represent our progress in creating an in vitro model of neocytolysis that would allow the dissection of underlying mechanisms.

In this report we demonstrated that human splenic endothelial cells possess EPO receptors, but only at observable levels after having been exposed to EPO, which indicates that receptor expression may be regulated by exposure to its ligand. When compared with endothelial cells from other organ sources, the monolayer of splenic cells uniquely responded to EPO withdrawal by increasing their permeability to an indicator molecule, fluoresceinated inulin. This process was also accompanied by morphological changes. For example, there were much greater numbers of pinocytotic vesicles and possible vesiculo-vacuolar organelles in EPO-withdrawn cells than in untreated cells. Vesiculo-vacuolar organelles are clusters of vesicles and vacuoles that traverse endothelial cells and provide transcellular pathways for macromolecules.[19] The presence of such structures may explain the increased permeability of the EPO-withdrawn cells.

When cultured alone, neither HSEC nor macrophages interacted significantly with red cells. When cultured with HSEC, the macrophages could ingest red cells. More interactions with old red cells than young red cells occurred in cultures kept uniformly with or without EPO; however, EPO withdrawal, while not affecting the level of old red-cell uptake, increased young red-cell uptake to the level of old red-cell ingestion. Thus, whether EPO was present or not, the major function of the endothelial cell–macrophage interaction was to remove old red cells, concomitant with what normally occurs in vivo. Only under the condition of EPO withdrawal did the additional function of young red-cell removal occur.

Young red cells normally interact in the spleen with macrophages, which aid in the removal of Howell-Jolly bodies, siderotic granules, and other inclusions from the

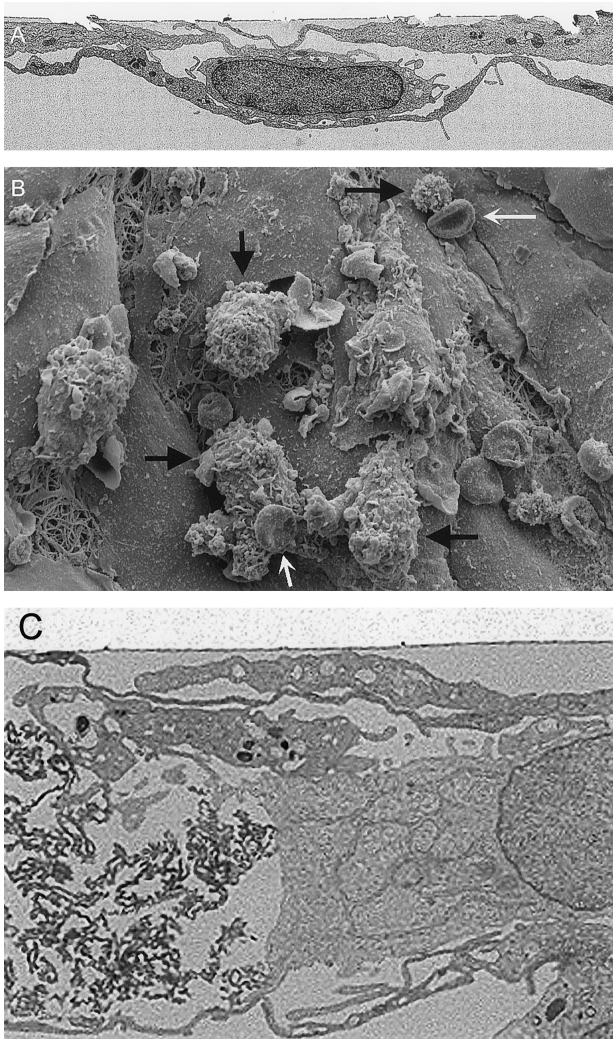

Figure 11. *Electron microscopy of cultures with HSEC, macrophages, and young red blood cells. HSEC and macrophages were grown for 72 hours with EPO and for 24 hours without. Panel A shows a transmission electron micrograph of an HSEC–macrophage coculture on glass. Panel B shows a scanning electron micrograph of a coculture on a collagen pad that had been EPO-withdrawn for 24 hours. Sorted young red cells were added to the culture for 1 hour before preparation for microscopy. Panel C is a transmission electron micrograph of a coculture that had been EPO-withdrawn for 24 hours. Sorted young red cells were added for 24 hours before preparation for microscopy.*

neocytes.[20] Our data support the possibility that the spleen is also the site for neocytolysis. Under conditions of EPO withdrawal, the normal processing of the young red cells by macrophages may change to destruction. Nonetheless, macrophages do not have EPO receptors, and so it is possible that signals that dictate the outcome of neocyte–macrophage interactions are transduced through adjacent endothelial cells that do have such receptors.[13] In the presence of EPO, endothelial cells may maintain associated macrophages in a state conducive to red cell processing by secreting growth factors or cytokines such as TGF-β.[21] EPO withdrawal from the endothelial cell may activate macrophages to phagocytose young red cells. Evidence for such activation of macrophages may be the increased production of proinflammatory cytokines in chronic anemia, which is associated with shortened red-cell survival.[22] Macrophages in turn can affect endothelial cells by producing vascular endothelial growth factor,[23] which, in addition to increasing endothelial cell permeability, induces the formation of pores through which red cells and platelets can extravasate in vivo.[24] Serum levels of vascular endothelial growth factor double during the first 3 days of space flight while EPO levels are decreased,[25] which may reflect a causal relationship between these changes.

Planned studies in our in vitro model will investigate the cytokine signals used in endothelial cell–macrophage communication and the adhesion molecules involved in macrophage–red cell interactions. We have also initiated an in vivo model of neocytolysis based on EPO-induced mild polycythemia in normal volunteers, followed by EPO withdrawal. Implications of these models extend from countermeasures for post-spaceflight anemia and for deadaptation from high altitudes, to optimizing therapeutic EPO dosing and increasing treatment options in polycythemias and hemolytic anemias.

ACKNOWLEDGMENTS

Our sincere thanks to Dr Cora Bucana and Kenneth Dunner, Jr, at the High Resolution Electron Microscopy Facility, University of Texas MD Anderson Cancer Center (supported by Institutional Core Grant #CA16672) for outstanding work and advice. We would also like to thank Fatemeh Moormehei and Michael Brown for excellent technical assistance, and Dr Terry Timme for assistance with immunofluorescence microscopy.

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