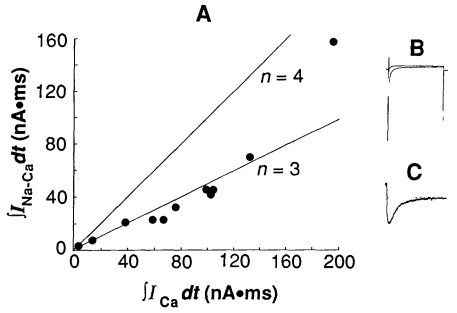

Fig. 3. (**A**) Relationship between the integral of I_{Ca} (abscissa) and the integral of $I_{Na\text{-}Ca}$ (ordinate). Each point represents measurements from a different cell. Under apparent steady-state conditions, successive trials on each cell were signal-averaged. (**B**) The integral of I_{Ca} was obtained by integrating the nifedipine sensitive current as this was assumed to reflect Ca^{2+} entry. Nifedipine (10 μM) sometimes caused a very small change in holding current (Ca^{2+} channel blockers are known to effect outward current) (*24*), which was neglected during the integration procedure. (**C**) The $I_{Na\text{-}Ca}$ was integrated for 2 s beneath the broken baseline. This typically included all the transient exchange and was assumed to reflect Ca^{2+} extrusion.

dependent current is a nonspecific current that is activated when $[Ca^{2+}]_i$ is elevated, since the available evidence indicates that nonspecific currents are just as effectively carried by Li^+, which we used as a replacement ion (*18, 19*). It is only when Na_o^+ is added in the presence of elevated $[Ca^{2+}]_i$ that $I_{Na\text{-}Ca}$ can be activated. Our integral of $I_{Na\text{-}Ca}$ was obtained by subtracting the stable current that was present after the transient exchange when the cell had mechanically relaxed. Whatever the origin of this stable current, it is unlikely to contain measurable inward $I_{Na\text{-}Ca}$ at resting values of $[Ca^{2+}]_i$ (that is, $\leq$153 nM) (*10*). Provided this stable current does not change significantly with $[Ca^{2+}]_i$, our integral should provide a reliable estimate of Ca^{2+} extrusion via Na^+-Ca^{2+} exchange. Our approach suggests that transient Ca^{2+} entry (I_{Ca}) and exit ($I_{Na\text{-}Ca}$) can be separated. This approach does not, however, allow us to separate steady-state Ca^{2+} entry (leak) from steady-state extrusion by the exchanger although, as we have already mentioned, some results suggest that the latter may be small (*10*).

Our results suggest that rapid perfusion methods may be used to separate I_{Ca} and transient $I_{Na\text{-}Ca}$ in ventricular cells. They also support earlier suggestions that prolonged Ca^{2+} current measurements in the presence of an inwardly directed Na^+ gradient (when $[Ca^{2+}]_i$ is elevated) will be contaminated by contributions from $I_{Na\text{-}Ca}$ (*20–23*). In addition to its importance in understanding transsarcolemmal Ca^{2+} extrusion, a clear separation of I_{Ca} and $I_{Na\text{-}Ca}$ is likely to be of value in any attempt to investigate possible "triggers" for Ca^{2+}-induced Ca^{2+} release that might underlie excitation contraction coupling in heart (*24*).

REFERENCES AND NOTES

1. J. H. B. Bridge, K. W. Spitzer, P. R. Ershler, *Science* **241**, 823 (1988).
2. D. Bers and J. Bridge, *Circ. Res.* **65**, 334 (1989).
3. T. Kitazawa, *J. Physiol. (London)* **402**, 703 (1988).
4. L. Barcenas-Ruiz, D. J. Beuckelmann, W. G. Wier, *Science* **238**, 1720 (1987).
5. K. Spitzer and J. Bridge, *Am. J. Physiol.* **256**, C441 (1989).
6. A. Hodgkin, P. McNaughton, B. Nunn, *J. Physiol. (London)* **358**, 447 (1985).
7. O. Hamill, A. Marty, E. Neher, B. Sakmann, F. Sigworth, *Pfleugers Arch.* **395**, 6 (1981).
8. B. Steadman, K. Moore, K. Spitzer, J. Bridge, *IEEE Trans. Biomed. Eng.* **35**, 264 (1988).
9. J. Kimura, S. Miyaumae, A. Noma, *J. Physiol. (London)* **384**, 199 (1987).
10. T. Ehara *et al.*, *ibid.* **410**, 227 (1989).
11. K.-W. Yau and K. Nakatani, *Nature* **311**, 661 (1984).
12. D. Noble, in *Excitation and Regulation of Contraction*, R. Nathan, Ed. (Academic Press, New York, 1986), p. 171.
13. A. Fabiato, *Am. J. Physiol.* **245**, C1 (1983).
14. B. Pitts, *J. Biol. Chem.* **254**, 6232 (1979).
15. J. Reeves and C. Hale, *ibid.* **259**, 7733 (1984).
16. J. H. B. Bridge and J. B. Bassingthwaighte, *Science* **219**, 178 (1983).
17. S. Wakabayashi and K. Goshima, *Biochem. Biophys. Acta* **642**, 158 (1981).
18. G. Yellen, *Nature* **296** (1982).
19. T. Ehara, A. Noma, K. Ono, *J. Physiol. (London)* **403**, 117 (1988).
20. L. Mullins, *Am. J. Physiol.* **236**, C103 (1976).
21. M. Mitchell, T. Powell, D. Terrar, V. Twist, *Br. J. Pharmacol.* **81**, 543 (1984).
22. D. Eisner and W. Lederer, *Am. J. Physiol.* **248**, C189 (1985).
23. J. Hume, *ibid.* **252**, H666 (1987).
24. B. London and J. Kreuger, *J. Gen. Physiol.* **88**, 4 (1986).

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Erythropoietin Retards DNA Breakdown and Prevents Programmed Death in Erythroid Progenitor Cells

MARK J. KOURY AND MAURICE C. BONDURANT

The mechanism by which erythropoietin controls mammalian erythrocyte production is unknown. Labeling experiments in vitro with [^{3}H]thymidine demonstrated DNA cleavage in erythroid progenitor cells that was accompanied by DNA repair and synthesis. Erythropoietin reduced DNA cleavage by a factor of 2.6. In the absence of erythropoietin, erythroid progenitor cells accumulated DNA cleavage fragments characteristic of those found in programmed cell death (apoptosis) by 2 to 4 hours and began dying by 16 hours. In the presence of erythropoietin, the progenitor cells survived and differentiated into reticulocytes. Thus, apoptosis is a major component of normal erythropoiesis, and erythropoietin controls erythrocyte production by retarding DNA breakdown and preventing apoptosis in erythroid progenitor cells.

ERYTHROPOIETIN (EPO), A GLYCOPROTEIN produced in the mammalian kidney and liver, controls erythrocyte production by acting directly on erythroid progenitor cells in the hematopoietic organs. Possible modes of Epo action include stimulation of mitosis, induction of a program of terminal differentiation, or maintenance of cellular viability. Eaves *et al.* (*1*) speculated that Epo maintains the viability of late-stage erythroid progenitors. Studies with purified, late-stage erythroid progenitor cells indicate that they require Epo for survival, notwithstanding any effects on mitosis or differentiation (*2, 3*). The Epo-dependent period extends from at least the colony-forming unit–erythroid (CFU-E) stage (*4*) to the stage at which hemoglobin synthesis begins (*2*).

To study the mechanism of Epo action, we used a homogeneous population of erythroid progenitor cells in the Epo-dependent period of development; the cells were isolated from spleens of mice infected with the anemia-inducing strain of Friend leukemia virus (FVA cells) (*5*). The Epo dependence and Epo-mediated differentiation of FVA cells in vitro were similar to those of CFU-E from normal, uninfected mice (*2, 5*). In earlier work, we observed no difference in DNA synthesis rates, but we did find a differential DNA breakdown in FVA cells cultured with Epo and those cultured without Epo (*2*). FVA cells cultured without Epo contained broken DNA, which was detected by 8 hours and was a prominent component by 16 hours. Because DNA breakage in Epo-deprived cells was observed only after 8 hours of culture, no conclusions could be drawn as to whether DNA break-

Division of Hematology, Vanderbilt University Medical Center and Veterans Administration Medical Center, Nashville, TN 37232.

age caused cell death or was merely an indicator of prolonged Epo deprivation.

We now present evidence that DNA cleavage is a characteristic of late erythroid progenitor cells and that Epo retards this cleavage. Because Epo retards DNA breakage and prevents cell death in cultured FVA cells, we suggest that Epo controls erythrocyte production by retarding DNA cleavage, thereby altering the course of erythroid progenitor cells toward death.

Labeling was done with [^{3}H]thymidine, and size analyses of the labeled cellular DNA were performed after various periods of subsequent culture. Labeled cells were washed and recultured with 20 μM thymidine (dT) plus 20 μM deoxycytidine (dC) (to prevent further incorporation of labeled material) or with 500 μM dT alone. Under the former conditions, DNA synthesis continues in FVA cells, while, at 500 μM dT or greater, DNA synthesis is inhibited via the thymidine block mechanism (*6*).

The survival of the cells under the various culture conditions reflected the fate of their DNA. In the presence of 500 μM dT, FVA cells, whether cultured with or without Epo, failed to differentiate and died, similar to unlabeled cells cultured without Epo (*2*). With 20 μM dT plus 20 μM dC, DNA synthesis was not inhibited and cells cultured with Epo differentiated and proliferated normally. FVA cells recultured without Epo showed a loss of labeled, high molecular weight DNA and an accumulation of labeled low molecular weight DNA fragments of 5000 bases or less (Fig. 1, B and D). Breakdown of labeled, high molecular weight DNA was also found in FVA cells cultured with Epo (Fig. 1, A and C), but it was less than in cultures lacking Epo.

With 20 μM dT plus 20 μM dC, FVA cells cultured without Epo had only 25% of labeled DNA remaining in uncleaved, high molecular weight form by 20 hours, whereas cells cultured with Epo had 66% of labeled DNA in this uncleaved form (Fig. 1E). These distributions of high and low molecular weight forms of DNA are similar to those found in unlabeled DNA from FVA cells cultured with and without Epo (*2*). When DNA synthesis was inhibited by 500 μM dT (Fig. 1E), the DNA breakdown was faster. However, the retardation of breakdown by Epo was still present, indicating that the effect of Epo is on cleavage rate and not on the rate of repair. A comparison of the DNA cleavages over the first 8 hours in culture with 500 μM dT (Fig. 1E) showed that the DNA cleavage was 2.6 times greater without Epo than with it. Thus, under conditions permissive for DNA synthesis, FVA cells without Epo have a rate of DNA cleavage in cultures that is sufficiently rapid to lead to net DNA breakdown, whereas in cultures with Epo the rate is retarded such that the majority of high molecular weight DNA is maintained intact. Under conditions of DNA synthesis inhibition, DNAs of cells with or without Epo suffer net breakdown but Epo still retards the cleavage rate.

To demonstrate that DNA breakdown is not an artifact of treatment that would occur with any cells, we used labeled mouse 3T3 cells (Balb/3T3). These cells have a doubling time and S-phase duration that are similar to those of FVA cells cultured in the presence of Epo (*2, 6, 7*). High molecular weight DNA was stable in Balb/3T3 cells in the log phase of growth even in the presence of 500 μM dT (Fig. 1E).

To determine whether the ^{3}H-labeled, lower molecular weight DNA fragments observed in alkaline denaturing gels were the result of single- or double-stranded cleavages and to determine more precisely their size range, we separated the DNAs from FVA cells on neutral, nondenaturing agarose gels 8 hours after they were labeled (Fig. 2, A to D). Much of the labeled DNA migrated as low molecular weight fragments, with apparent sizes between 100 and 5000 bp. The sizes of these lower molecular weight DNA fragments were unchanged after digestion with S_1 nuclease, which specifically cleaves single-stranded DNA (*6*). These results indicate that the small DNA fragments were present intracellularly as double-stranded fragments.

Although alkaline agarose gels (Fig. 1) provided good separations of high and low molecular weight DNA, they were unable to provide adequate resolutions of DNA fragments below 2 kb in length. The neutral agarose gels (Fig. 2, A to D) showed that fragments between 0.1 and 1 kb were present in the cells. For more precise size determination, polyacrylamide-urea gel separations were performed (Fig. 2, E and F). These gels showed a periodicity in the size of labeled cleaved DNA. The lowest molecular weight fragments were between 150 and 200 bases long, with multiples of the smallest unit being evident in larger fragments. The size of this smallest unit is consistent with the length of DNA associated with nucleosomes (*8*). The size and distri-

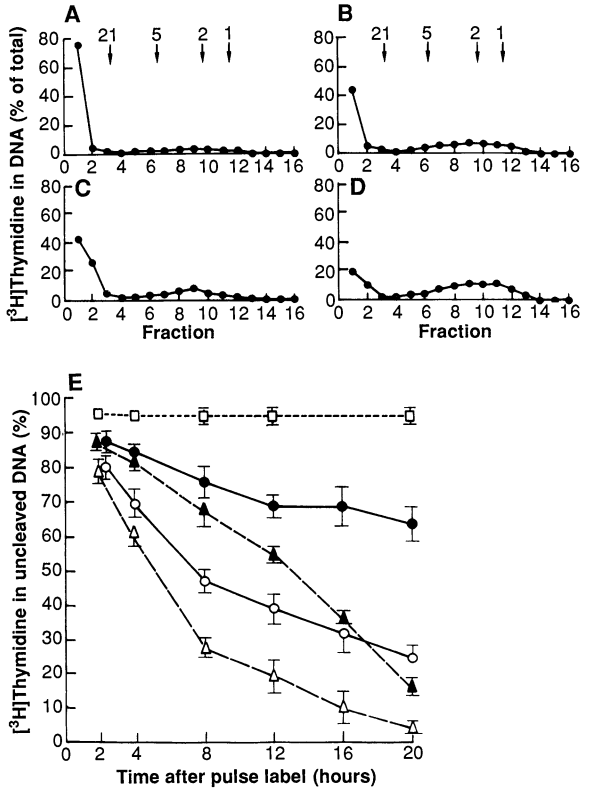

Fig. 1. Effect of Epo on the rate of DNA cleavage in FVA cells. Splenic erythroid progenitor cells (FVA cells) were isolated from CD_2F_1 mice infected with the anemia-inducing strain of Friend leukemia virus and were cultured in liquid medium as described (*2, 5*). After 30 min, [^{3}H]thymidine (0.2 μCi/ml; 6.7 Ci/mmol) was added to the medium and the cells were incubated for a further 30 min. The cells were washed twice and recultured in fresh medium with 20 μM unlabeled dT plus 20 μM dC or with 500 μM dT alone. At the time of reculture, pure recombinant human Epo (150 milliunits/ml) was added to half of the cultures. Balb/3T3 cells in log phase growth were similarly labeled. At various times after reculture, the cells were harvested and their DNA was extracted (*2*). The DNA was electrophoretically separated on alkaline, 0.6% agarose gels (*16*). Gel lanes were cut into 5-mm fractions beginning at the origin, and the radioactivity in each fraction was expressed as a percentage of the total radioactivity in each lane. The distribution of [^{3}H]thymidine in DNA is shown for FVA cells cultured for 8 hours (**A**) with Epo plus 20 μM dT and 20 μM dC, (**B**) without Epo plus 20 μM dT and 20 μM dC; (**C**) with Epo plus 500 μM dT; and (**D**) without Epo plus 500 μM dT. Numbers across the top of the figure indicate the migration of DNA marker fragments of the number of kilobases shown. (**E**) The percentage of [^{3}H]thymidine in high molecular weight, uncleaved DNA in FVA cells at various times of culture with Epo plus 20 μM dT and 20 μM dC (●); without Epo plus 20 μM dT and 20 μM dC (○); with Epo plus 500 μM dT (▲); and without Epo plus 500 μM dT (△). The results with Balb/3T3 cells plus 500 μM dT (□) were the same as those with Balb/3T3 cells plus 20 μM dT and 20 μM dC. The combined percentages of [^{3}H]thymidine in fractions 1 and 2 of alkaline agarose gels were considered to be the uncleaved percentage at each time. Data are ±1 SD from three separate experiments.

bution of labeled cleaved DNA fragments in FVA cells cultured with Epo were similar to those in cells cultured without it. The only difference was the faster conversion to low molecular weight fragments in FVA cells cultured without Epo. DNA from unlabeled FVA cells cultured for 20 hours with or without Epo had similar patterns of breakdown (Fig. 2F).

To ensure that the observed DNA instability was not due to Friend leukemia virus infection, we cultured enriched erythroid progenitor cells from the spleens of anemic uninfected mice with and without Epo. Pulse-labeling experiments in these cells showed that Epo decreased DNA breakdown (Fig. 3). The rates and extent of labeled DNA breakdown in the uninfected cells were less than those found at the same times in the corresponding FVA cell cultures (compare Fig. 3 with Fig. 1E). These differences most likely occur because the uninfected cell population contains significant percentages of nonerythroid cells and of erythroid progenitor cells that were developmentally either before or after the Epo-dependent stages. Also, these anemic mice have thousands of milliunits of Epo per milliliter of serum (*9*), whereas FVA-infected mice have 16 milliunits/ml, and normal mice have 35 milliunits/ml (*10*). The high Epo levels in the anemic mice are sufficient to saturate Epo receptors on the splenic erythroid cells in vivo, and thus an effect of residual endogenous Epo on these cells may alter their DNA cleavage rates compared to FVA cells. An immunological response to the FVA cells is another possible cause for the observed differences. However, this is less likely because the culture medium, which contains heat-inactivated fetal bovine serum, lacks functional complement proteins that are needed for antibody-mediated cytotoxicity, and the FVA cell population, of which 95% or more is made up of erythroblasts (*5*), has few cells that can function as cytotoxic effector cells.

The increased rate of DNA breakdown in FVA cells cultured without Epo preceded a series of events that included accumulation of small DNA fragments, the absence of cell division, unresponsiveness to Epo, and, eventually, cell death (*2*). By trypan blue dye exclusion, some FVA cells cultured without Epo began to die between 12 and 16 hours, although most of them survived for 24 hours (*2*). In contrast, FVA cells cultured with Epo survived and differentiated into reticulocytes within 48 hours (*2, 5*). The mechanism whereby Epo modulates the rate of DNA cleavage and consequent breakdown in erythroid progenitors is unclear. This modulation does not depend on adenosine triphosphate concentrations, which are the same in FVA cells cultured with Epo as in those cultured without Epo for at least 6 hours (*11*). A newly synthesized or a short-lived protein may be involved in the Epo modulation because the protein synthesis inhibitor cycloheximide retards the breakdown of DNA in FVA cells (*6*).

Intranucleosomal cleavage of DNA, inhibition of such cleavage by cycloheximide, maintenance of intracellular energy and impermeability to trypan blue are characteristic of cells undergoing programmed cell death (apoptosis) (*8, 12*). Thus, during terminal differentiation, mammalian erythroid progenitor cells undergo a process of DNA cleavage that has several characteristics of apoptosis. In the absence of sufficient Epo, this process is rapid and the cells die. In the presence of sufficient Epo, the process is

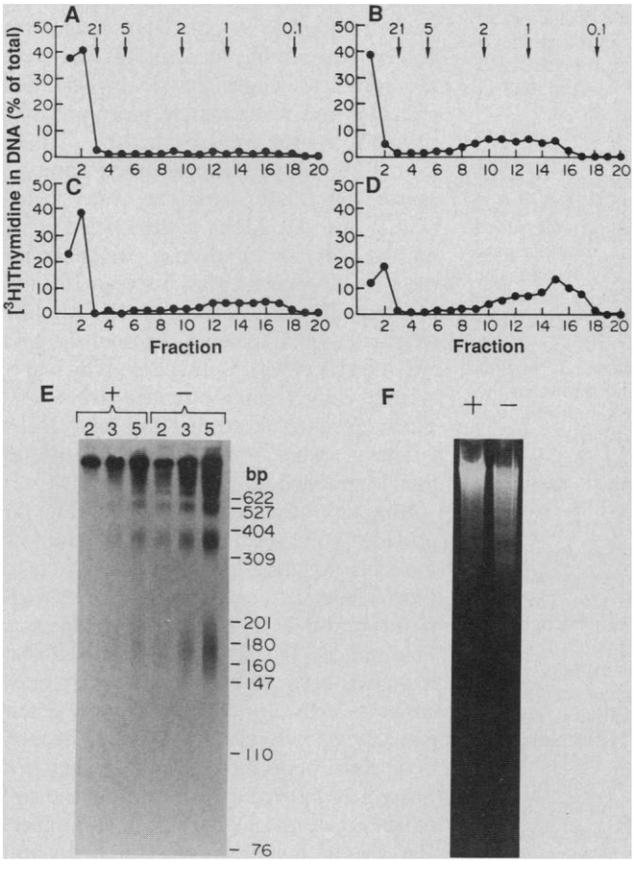

Fig. 2. Size characteristics of lower molecular weight DNA in FVA cells. (**A** to **D**) The same 8-hour samples of DNA from FVA cells as shown in Fig. 1 (A to D), respectively, were electrophoretically separated as described in the legend to Fig. 1, except that neutral *p*H gels were used. The gel lanes were cut into 5-mm fractions, and radioactivity per fraction was expressed as a percentage of total radioactivity. Numbers at top of figure indicate the migration of DNA fragments of the number of kilobase pairs shown. (**E**) FVA cells were radiolabeled and cultured with (+) or without (−) Epo, as described in the legend to Fig. 1, with [^{3}H]thymidine (3 μCi/ml) followed by unlabeled dT (500 μ*M*) for 4 hours. DNA samples from these cells were electrophoretically separated on a 7*M* urea–8% polyacrylamide gel (*17*). The number above each lane represents the counts per minute (×10^4) of [^{3}H]thymidine in DNA that was loaded. The gel was fixed and processed for fluorography, and an autoradiograph was made by exposure for 21 days at −80°C. Markers indicate migration of DNA fragments of number of bases shown. (**F**) Ethidium bromide staining of DNA samples from unlabeled FVA cells cultured for 20 hours with Epo (+) or without Epo (−), which were separated on a 7*M* urea–5% polyacrylamide gel. Each lane was loaded with 6 μg of DNA.

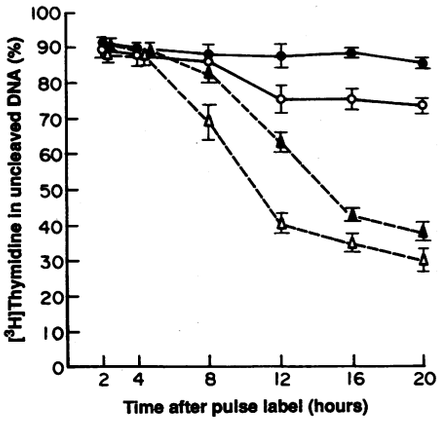

Fig. 3. DNA cleavage in spleen erythroid cells from mice with short-term anemia. CD_2F_1 mice had their hematocrits reduced from the normal 49 to 50% to 20 to 23% by bleeding 0.6 ml at 40 and 36 hours before they were killed (*9*). The splenic cells were then separated by velocity sedimentation at unit gravity in the same manner as FVA cells (*5*) to further enrich the erythroid precursors. Wright-stained cytocentrifuge preparations showed that 42 to 50% of the nucleated cells were erythroid. Proerythroblasts and basophilic erythroblasts were predominant among the erythroid cell populations. These cells were cultured and labeled as described in the legend to Fig. 1. The DNA was extracted and separated on alkaline agarose gels, and [^{3}H]thymidine in uncleaved DNA was plotted as a function of time as described in Fig. 1E; (●) cells cultured with Epo plus 20 μ*M* dT and 20 μ*M* dC; (○) cells cultured without Epo plus 20 μ*M* dT and 20 μ*M* dC; (▲) cells cultured with Epo plus 500 μ*M* dT; and (△) cells cultured without Epo plus 500 μ*M* dT.

retarded and the progenitor cells survive and subsequently develop into erythrocytes.

Studies in vivo showed that low Epo concentrations led to decreased CFU-E numbers and high Epo concentrations led to increased CFU-E numbers, but the percentages of CFU-E in DNA synthesis were similar at all Epo levels (*13*). Studies in vitro showed that maximum CFU-E growth requires Epo concentrations which are several times higher than those of normal serum (*14*) and that almost all CFU-E are lost after a few hours in culture without Epo (*15*). Our data and these previous studies support a model of erythrocyte production in which Epo facilitates survival of progenitor cells. In this model, erythroid progenitor cells have a period during development in which they require Epo to prevent their death. This Epo-dependent period includes at least the stages of CFU-E and their immediate descendants, the proerythroblasts. The model predicts that during normal erythropoiesis, Epo levels in vivo are insufficient for the survival of a majority of the Epo-dependent progenitors. Thus, a minority of the Epo-dependent erythroid progenitors are responsible for normal erythrocyte production. When Epo levels are increased because of anemia or hypoxia, many of the Epo-dependent progenitors that would ordinarily die in the presence of normal Epo levels will survive, differentiate, give rise to reticulocytes, and thereby increase erythrocyte production. Conversely, when Epo levels are decreased below normal as a result of hypertransfusion, some of the relatively small percentage of Epo-dependent progenitors that would survive in the presence of normal Epo levels will die because of inadequate Epo supply, and thus erythrocyte production will be reduced.

REFERENCES AND NOTES

1. C. J. Eaves, R. K. Humphries, G. Krystal, A. C. Eaves, *Hemoglobins in Development and Differentiation*, G. Stamatoyannopoulos and A. W. Nienhuis, Eds. (Liss, New York, 1981), p. 63.
2. M. J. Koury and M. C. Bondurant, *J. Cell. Physiol.* **137**, 65 (1988).
3. K. T. Landschulz, A. N. Noyes, O. Rogers, S. H. Boyer, *Blood* **73**, 1476 (1989).
4. J. R. Stephenson, A. A. Axelrad, D. L. McLeod, M. M. Shreeve, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1542 (1971).
5. M. J. Koury, S. T. Sawyer, M. C. Bondurant, *J. Cell. Physiol.* **121**, 526 (1984); S. T. Sawyer, M. J. Koury, M. C. Bondurant, *Methods Enzymol.* **147**, 340 (1987).
6. M. J. Koury and M. C. Bondurant, unpublished data.
7. J. Campisi and A. B. Pardee, *Mol. Cell. Biol.* **4**, 1807 (1984).
8. A. H. Wyllie, *Nature* **284**, 555 (1980); ———, R. G. Morris, A. L. Smith, D. Dunlop, *J. Pathol.* **142**, 67 (1984).
9. S. T. Koury, M. J. Koury, M. C. Bondurant, J. Caro, S. E. Graber, *Blood* **74**, 645 (1989).
10. The low concentrations of Epo in mice infected with anemia-inducing Friend disease are due to a moderate virus-induced increase in blood erythrocytes, which is masked by a concomitant increase in plasma volume [P. E. Tambourin *et al.*, *Br. J. Haematol.* **24**, 511 (1973)].
11. M. J. Koury and H. D. Kim, unpublished data.
12. A. H. Wyllie, J. F. R. Kerr, A. R. Currie, *Int. Rev. Cytol.* **68**, 251 (1980).
13. N. N. Iscove, *Cell Tissue Kinet.* **10**, 323 (1977).
14. C. J. Gregory, A. D. Tepperman, E. A. McCulloch, J. E. Till, *J. Cell. Physiol.* **84**, 1 (1974); C. J. Eaves and A. C. Eaves, *Blood* **52**, 1196 (1978).
15. N. N. Iscove, in *Hematopoietic Cell Differentiation*, D. W. Golde, M. J. Cline, D. Metcalfe, C. F. Fox, Eds. (Academic Press, New York, 1978), p. 37; J. M. Lipton, M. Kudish, D. G. Nathan, *Exp. Hematol.* **9**, 1035 (1981); D. F. Del Rizzo and A. A. Axelrad, *ibid.* **13**, 1055 (1985).
16. T. Maniatis, E. F. Fritsch, J. Sambrook, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982), p. 171.
17. L. G. Davis, M. D. Dibner, J. F. Battey, *Basic Methods in Molecular Biology* (Elsevier, New York, 1986), p. 264.

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