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The rate of apoptosis in post mitotic neutrophil precursors of normal and neutropenic humans

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Abstract. Using data on the fraction of post-mitotic neutrophil precursors ($CD15^+$ cells) displaying positive markers for apoptosis in 12 normal humans, and a simple mathematical model, we have estimated the apoptotic rate to be about 0.28/day in this compartment. This implies that the influx of myelocytes into the post-mitotic compartment exceeds twice the granulocyte turnover rate (GTR), and that about 55% of the cells entering this compartment die before being released into the blood. The normal half life of apoptotic post-mitotic neutrophil precursors is calculated to be 10.4 h. Comparable calculations for patients indicate apoptosis rates in the post-mitotic compartment of about 17 times normal for one myelokathexis patient and rates of about 13 times normal for the one cyclical neutropenic patient and two severe congenital neutropenic patients. The estimated half life for apoptotic post-mitotic neutrophil precursors in the myelokathexis patient was about 0.4 h, 1.4 h in the cyclical neutropenia patient, and about 0.6 h in the severe congenital neutropenic patients.

INTRODUCTION

There are a variety of periodic haematological diseases in which statistically significant oscillations in the number of circulating blood cells (white blood cells, red blood cells, and/or platelets) are observed, with periods ranging from days to weeks to months (Haurie *et al*. 1998). Some of these involve the oscillation of only a single cell type and are probably due to the destabilization of a peripheral cell production control mechanism, e.g. periodic auto-immune haemolytic anaemia (Bélair *et al*. 1995; Mahaffy *et al*. 1998) and cyclical thrombocytopenia (Santillán *et al*. 2000; Swinburne & Mackey 2000). Others involve oscillations of the number of all of the circulating blood cell types with the same period, though out of phase with one another. Two examples are periodic chronic myelogenous leukaemia (Fortin & Mackey 1999) and cyclical neutropenia (Haurie *et al*. 1999).

Arguably the best studied and characterized of these periodic haematological diseases is cyclical neutropenia. In humans the period ranges from 11 to 52 days with the preponderance of patients having periods in the range of 20–30 days (Haurie *et al*. 1998). Our understanding

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of cyclical neutropenia in humans has been greatly aided by the existence of the grey collie animal model. Cyclical neutropenia in the grey collie is qualitatively identical with the condition in humans except for the period (Haurie *et al*. 1999b).

Early mathematical modelling of cyclical neutropenia dynamics (Mackey 1978, 1979) led to the hypothesis that cyclical neutropenia was due to an abnormally high level of cell death at the stem cell level, leading to a bifurcation (qualitative change) in the dynamics of neutrophil production and the ensuing oscillations. This led to further predictions (Mackey 1996) concerning the results of decreasing this level of apoptosis which have received circumstantial confirmation both clinically (Haurie *et al*. 1999a) and experimentally (Haurie *et al*. 1999b) through the use of granulocyte colony stimulating factor (G-CSF). This work also predicted a strong link between cyclical neutropenia and other forms of neutropenia (e.g. severe congenital neutropenia, Haurie *et al*. 1999a). The recent demonstration of elevated numbers of apoptotic cells in the bone marrow of cyclical neutropenic patients (Aprikyan *et al*. 2001) lends more direct support to the hypothesis that cyclical neutropenia is a consequence of elevated levels of apoptosis.

If indeed the neutropenias are due to increased apoptosis rates, then it is clearly of importance to quantify these rates in normal individuals as well as in neutropenic patients. Here we use normal and neutropenic patient data from Aprikyan *et al*. (2000, 2001) to calculate the quantitative rate of apoptosis in post mitotic neutrophilic precursors, as well as the rate of disappearance of apoptotic cells.

DATA AND ANALYSIS TECHNIQUES

Purification of bone marrow progenitor cells

Bone marrow mononuclear cells were isolated from bone marrow aspirates by modification of previously described methods (Park *et al.* 2000). The resultant cells from patients and healthy donors were fractionated into $CD34^+$ early progenitors, $CD33^+/CD34^-$ myeloid progenitors and $CD15^+/CD33^-/CD34^-$ bone marrow granulocyte precursor subpopulations using cell surface antigen-specific monoclonal antibodies and immunomagnetic beads from Miltenyi Biotech Inc. (Auburn, CA, USA), according to the manufacturer's recommendations. Purity of each bone marrow haematopoietic subpopulation was greater than 96% as tested by FACS analysis (data not shown). All of these studies were performed after obtaining informed consent from the patients and the normal subjects.

Apoptosis assays

Annexin V binding to neutrophils and bone marrow progenitor cells was performed using an apoptosis detection kit (R & D Systems, Minneapolis, MN, USA). Briefly, $3–20 \times 10^4$ freshly isolated cells or cells cultured overnight at 37 °C in a 5% CO_2-incubator in RPMI (BioWhittaker, Walkersville, MD, USA) in the presence of 10% autologous serum were labelled with FITC-conjugated annexin V and propidium iodide (PI) for 20 min at room temperature. The samples were washed twice, and analysed by two-colour flow cytometry using CellQuest Analysis software (Becton Dickinson, Mountain View, CA, USA). A minimum of 10 000 events was counted per sample. Results are reported as the percentage of annexin V positive cells, which reflects the relative proportion of apoptotic cells in early and late stages of apoptosis (Anthony *et al*. 1998; Albanese *et al*. 1999).

The apoptosis assay is based on the flow cytometry analysis of cells labelled with FITC-Annexin V and PI which allows detection of cells in early and late stages of apoptosis (Aprikyan *et al*. 2001; Vermes *et al*. 1995). If cells were undegoing necrosis, annexin V and PI-positive

cells would be expected, whereas the presence of FITC-annexin V-positive but PI-negative cells is indicative of apoptotic rather than necrotic cell death. Flow cytometry analysis revealed a negligible proportion (less than 1%) of PI-positive cells in patients' and control cell subpopulations regardless of whether the cells were freshly isolated or cultured overnight.

Analysis techniques

To estimate the extent of apoptosis in the post-mitotic compartment from the data, we have carried out a simple mathematical modelling exercise. Using the fraction of apoptotic post-mitotic cells (f_a), the total post-mitotic population (viable plus apoptotic, T), the duration of the post-mitotic compartment (τ_m), the density of circulating neutrophils (N), the rate of neutrophil removal from the circulation (α), and the granulocyte turnover rate (GTR), we are able to estimate the post-mitotic apoptotic rate (γ), the rate of removal of cells once they become apoptotic (δ), and the influx of cells from the myelocyte stage (V_0). The details are in the Appendix.

RESULTS

Normal controls

The results of the analysis for the 12 normal individuals are given in Table 1, and they predict $\gamma \simeq 0.28 \pm 0.2$/day. This implies that the ratio $[V_0/GTR] = [1/r(\gamma,\delta)]$ (see Appendix) of the myelocyte influx to the post-mitotic compartment to the efflux to the circulation (the GTR) is in the order of 2.3. This, in turn, means that about 55% of all cells that enter the post-mitotic pool are destined to die in normal individuals before being released into the circulation.

Table 1. Results of the analysis for 12 normal individuals. The fraction f_a of post-mitotic ($CD15^+$) apoptotic cells was determined as described in the text. The density of circulating neutrophils $N = 4.0 \times 10^8$ cells/kg, as well as the size of the post-mitotic compartment $T = 5.59 \times 10^9$ cells/kg was taken from Table III of Dancey *et al*. (1976). The duration of the post-mitotic compartment $\tau_m = 3.8$ days was taken from Hearn *et al*. (1998), who based their calculations on the data of Price *et al*. (1996). γ and δ (both in units/days) were determined from the simultaneous solution of Equations 6 and 8. Once γ and δ are determined, the myelocyte influx (V_0) (in units of 10^9 cells/kg/day) into the post-mitotic pool is determined from Equation 7. GTR = $\alpha N = 8.7 \times 10^8$ cells/kg/day is from Dancey *et al*. (1976). See the text for further details

Ctl.	f_a	γ	δ	V_0	$\frac{V_0}{GTR}$
1	0.11	0.27	1.96	2.08	2.38
2	0.12	0.27	1.78	2.07	2.36
3	0.13	0.27	1.62	2.05	2.34
4	0.11	0.27	1.96	2.08	2.38
5	0.18	0.30	1.13	2.01	2.30
6	0.20	0.31	1.00	1.99	2.28
7	0.14	0.28	1.49	2.05	2.34
8	0.10	0.26	2.18	2.09	2.39
9	0.09	0.26	2.45	2.11	2.41
10	0.17	0.29	1.20	2.01	2.30
11	0.21	0.31	0.95	1.99	2.27
12	0.14	0.28	1.50	2.05	2.34
Av.	0.14	0.28	1.60	2.05	2.34
SD	0.04	0.02	0.48	0.04	0.05

Table 2. Analysis results for the myelokathexis (MK), cyclical (CN) and severe congenital (SCN) neutropenic patients of this study. The fraction f_a of apoptotic cells was determined as before. f_N, the fraction of circulating neutrophils relative to normal was based on the neutrophil count determined for each individual and a value of 4.4×10^3 cells/mm^3 for normal individuals (Dancey *et al*. 1976). The fraction f_T of the post-mitotic marrow neutrophil precursor compartment (metamyelocytes and bands) was determined from actual bone marrow differentials on each patient and a normal value of 0.445 was determined from a weighted average of the figures of Dancey *et al*. (1976) (Table III, 43.2% of the total marrow neutrophilic cells were metamyelocytes and bands, $n = 13$) and the control values of Chatta *et al*. (1996) (Table 2, the post-mitotic pool constitutes 46.0% of total marrow neutrophils, $n = 13$). The duration of the post-mitotic compartment τ_m was taken to be 1.2 days from Hearn *et al*. (1998) for neutropenic patients. All else as in Table 1

Diag.	f_a	f_N	f_T	γ	δ	V_0	$\frac{V_0}{GTR}$
MK	0.10	0.089	1.02	4.85	43.6	23	298.1
CN	0.23	0.172	0.494	3.6	11.9	7.9	52.4
SCN	0.11	0.044	0.135	3.56	28.8	2.4	62.8
SCN	0.10	0.014	0.045	3.61	32.4	0.83	67.6

The second interesting feature of the analysis is the predicted rate of disappearance of apoptotic cells, $\delta \simeq 1.60 \pm 0.48$/day. This corresponds to a half life of apoptotic post-mitotic neutrophil precursors of $t_{1/2} = (\ln 2)/\delta = 0.43$ days = 10.4 h.

Patient results

The results of the analysis for the four patients studied are presented in Table 2. In brief, all showed apoptosis rates within the post-mitotic neutrophil presursors more than 10 times normal and correspondingly high rates of disappearance of apoptotic cells. The cyclical and severe congenital neutropenic patients had apoptotic rates γ that were virtually identical and about 13 times normal, and the myelokathexis patient had a significantly higher value of γ that was about 17 times normal. The half life for the post-mitotic apoptotic cells is estimated in the myelokathexis patient to be about 0.4 h, 1.4 h in the cyclical neutropenia patient, and about 0.6 h in the severe congenital neutropenic patients.

DISCUSSION

In several forms of neutropenia, both inherited and acquired, there is now good evidence that accelerated apoptosis of marrow progenitor cells is the primary pathophysiological mechanism reducing the blood neutrophil counts. In congenital and cyclic neutropenia, this abnormality occurs at the stage of commitment of multipotent stem cells to the neutrophil lineage (Aprikyan *et al*. 2001); in myelokathexis it occurs later in this process (Aprikyan *et al*. 2000). Accelerated apoptosis is also regarded now as the cause of neutropenia in patients with myelodysplasia Shetty *et al*. (2002).

However, to our knowledge there have not been any previous attempts to quantify the rate of apoptosis in either normal individuals or patients. There have, however, been indirect estimates of apoptosis rates in the stem cell populations of laboratory animals. Based on a stochastic mathematical modelling study, Abkowitz and colleagues obtained values (Abkowitz *et al*. 1996, 2000) for γ of 0.007 (range of 0–0.071)/day in cats, and γ between 0 and 0.034/day in mice. Mackey (2001) analysed the *in vivo* murine data of Bradford *et al*. (1997) and Cheshier *et al*. (1999) to obtain stem cell apoptosis rates γ of 0.069 (0, 0.20) and 0.228 (0, 0.599)/day, respectively.

More directly related to this study, Haurie *et al*. (2000) estimated that $0.1 \leq \gamma \leq 0.4$/day for committed neutrophil precursors based on the fit of a model for the regulation of neutrophil production to the cycling neutrophil levels in grey collies.

The range of these estimated values for γ in stem cells and the neutrophil lineage clearly points out the desirability of having other independent estimates of γ derived from more direct kinetic observation of apoptotic cells, which this study furnishes.

The level of apoptosis calculated here has interesting implications. As the normal input to the post-mitotic neutrophil compartment is calculated to be about 2.3 times the GTR (cf. Table 1), a reduction of the rate of apoptosis to zero would more that double the GTR. This offers an interesting insight into the compensatory mechanisms regulating neutrophil levels in situations of acute demand. For example, a substantial fall in neutrophil numbers would lead to an increased level of circulating G-CSF. This would, in turn, lead to a decreased level of apoptosis and consequent increase in the GTR. This elevated effective production of neutrophils would also be felt rapidly, as the increased levels of G-CSF would not only decrease the level of apoptosis but also decrease the time spent (τ_m) in the post-mitotic compartment, as has been documented by Chatta *et al*. (1994).

These considerations can be extended to the entire neutrophil production scheme. Hearn *et al*. (1998) have shown that the neutrophil transit times in humans can be accurately fit using the density of a gamma distribution. Haurie *et al*. (2000) used their mathematical model for the G-CSF regulation of peripherial neutrophil production to predict that the ratio of the maximum GTR to the steady state GTR should be given by

$$\Gamma \equiv \frac{\mathrm{GTR}_{\max}}{\mathrm{GTR}_{\mathrm{ss}}} = e^{\gamma \tau_m} \left(\frac{a + \gamma}{a} \right)^{m+1}, \tag{1}$$

where a and m are parameters characterizing the density of the neutrophil maturation times. For normal humans, Hearn *et al*. (1998) find $a = 0.364$/day and $m = 1.15$ from the data of Price *et al*. (1996). If γ has the same value in the proliferating neutrophil precursors as we have calculated for the post-mitotic precursors, then this would imply from Equation 8 that $\Gamma \simeq 9.88$.

This, in turn, implies that (under the assumptions stated above) the GTR in normal humans could be increased to a maximum of about 10 times the normal value simply by decreasing apoptosis to zero in all of the committed neutrophil precursors. This would amount to an additional effective three divisions within the entire lineage. This estimate of Γ is consistent with the estimate of $8 \leq \Gamma \leq 16$ based on theoretical grounds in Hearn *et al*. (1998) and the estimate of $\Gamma \geq 9.4$ for humans (Lord 1992; Lord *et al*. 1992) and $\Gamma \geq 14.5$ in mice (Lord *et al*. 1991).

The half lives of apoptotic cells we have estimated highlight the difficulty in studying pathology involving apoptotsis as it is likely, based on our estimates, that apoptotic cells are rapidly removed and therefore difficult to observe. We have no explanation for the differences in clearance rates of apoptotic cells between normal and pathological states.

Finally we note that exactly this same technique could be used to determine levels of apoptosis in post-mitotic leukaemic cell populations after chemotherapy.

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APPENDIX ON ANALYSIS TECHNIQUES

To estimate the extent of apoptosis in the post-mitotic compartment from the data, we have carried out a simple mathematical modelling exercise. We only consider post-mitotic neutrophil precursors cells (metamyelocytes, banded and segmented neutrophils). These post-mitotic cells have an input from the myelocytes, the last mitotic stage cells. The age of the post-mitotic cells when they enter from the myelocyte stage is taken to be $a = 0$, and the distribution of viable cells as a function of age is given by $V(a)$ (with dimension cells/kg/day). It is assumed that only viable (non-apoptotic) cells leave the myelocyte compartment for the post-mitotic compartment. The flux of viable myelocytes into the post-mitotic compartment is denoted by $V_0 \equiv V(a = 0)$. The maximum age in the post-mitotic compartment is given by τ_m, which has been determined in Hearn *et al*. (1998) from the data of Price *et al*. (1996).

Viable cells are assumed to disappear from the post-mitotic compartment in two ways. Firstly, they may become apoptotic at a rate γ during the ageing process. γ is one of the two parameters to be determined. However, if they do not become apoptotic before attaining the maximal age $a = \tau_m$ they are then released into the circulation. Thus, as $V(a)$ denotes the density of viable cells, their dynamics are described by the differential equation $dV/da = -\gamma V$ whose solution is $V(a) = V_0 e^{-\gamma a}$. The total number of viable post-mitotic cells of all ages is given by

$$\mathcal{V} \equiv \int_0^{\tau_m} V(a)da = \frac{V_0}{\gamma}[1 - e^{-\gamma\tau_m}] \equiv V_0 q(\gamma). \tag{2}$$

Further, post-mitotic apoptotic cells are assumed to arise only through the apoptotic conversion of viable cells (at a rate γ, see above), are degraded randomly at a rate δ, and if not degraded by age τ_m are released into the circulation as are viable cells. δ is the second parameter to be determined. If the density of apoptotic cells is denoted by $D(a)$ their dynamics are governed by $dD/da = \gamma V(a) - \delta D$, and their age distribution is given by $D(a) = V_0\gamma\{e^{-\gamma a} - e^{-\delta a}\}/(\delta - \gamma)$. The total number of apoptotic cells in the post-mitotic compartment is given by

$$\mathfrak{D} = \int_0^{\tau_m} D(a)\, da = V_0 p(\gamma,\delta) \tag{3}$$

where

$$p(\gamma,\delta) \equiv \frac{\gamma}{\delta - \gamma}\left\{\frac{1 - e^{-\gamma\tau_m}}{\gamma} - \frac{1 - e^{-\delta\tau_m}}{\delta}\right\} \tag{4}$$

Therefore, the total population (viable plus apoptotic) of post-mitotic cells is given by

$$\mathcal{T} = \mathcal{V} + \mathfrak{D} = V_0[p(\gamma,\delta) + q(\gamma)], \tag{5}$$

so the *fraction* of cells in the post-mitotic population that are apoptotic (and thus observable) is given by

$$f_a = \frac{\mathfrak{D}}{\mathcal{T}} = \frac{p(\gamma,\delta)}{p(\gamma,\delta) + q(\gamma)}. \tag{6}$$

Equation 6 is an equation involving two unknowns (γ and δ) which are to be determined, and two known quantities (f_a determined experimentally, and τ_m as determined by Hearn *et al*. (1998)). To determine γ and δ we therefore require a second equation involving these two quantities.

Let N (cells/kg) be the density of circulating neutrophils. Neutrophils are eliminated from the circulation randomly with a $t_{1/2} = 7.6$ h corresponding to a rate $\alpha = 24 \times (\ln 2)/t_{1/2} \simeq 2.2$/day (Dancey *et al*. 1976), so the flux of neutrophils to death (the granulocyte turnover rate, GTR) is given by αN (cells/kg/day). In a steady state, this flux must be equal to the input of neutrophils from the post-mitotic bone marrow compartment:

$$\mathrm{GTR} = \alpha N = V(\tau_m) + D(\tau_m) \equiv V_0 r(\gamma,\delta). \tag{7}$$

Eliminating V_0 between Equations 5 and 7 yields finally

$$\frac{\alpha N}{T} = \frac{r(\gamma,\delta)}{p(\gamma,\delta) + q(\gamma)}. \tag{8}$$

This is the second relation desired involving γ and δ as all other quantities on the left hand side of Equation 8 are known (Dancey *et al*. 1976) for normal humans.