A central problem of the current studies on protooncogenes (cellular oncogenes, c-onc) is their role in the control of growth and differentiation of normal cells. The c-fos protooncogene has been widely investigated and it has been demonstrated that c-fos specific transcripts are induced in proliferating cells (1, 2) and during differentiation (3–6). In particular, when HL60 (promyelocytic) and U937 (promonocytic cell line) are induced to differentiate to cells with the characteristics of monocytes/macrophages by treatment with PMA or IFN-γ (4–6), c-fos mRNA is detectable within 10 min. Similarly, when the murine myelomonocytic leukemia WEHI-3B was induced to differentiate to monocytes by granulocyte colony-stimulating factor (GCSF) and Actinomycin D, expression of c-fos was seen (4). Along the same line, when human myeloid leukemias at different stages of differentiation were examined, c-fos transcripts were only detectable in the more mature monocytic forms (7). Moreover, freshly isolated murine peritoneal macrophages express appreciable levels of c-fos-specific mRNA, which can be augmented by endotoxin (8) and colony-stimulating factor 1 (CSF-1) (4). Thus, although expression of c-fos may be neither sufficient nor obligatory for the differentiation of mononuclear phagocytes (6), and it has been suggested (6) that c-fos expression plays a role only in growth arrest of immature myeloid elements, its consistent association with mononuclear phagocytes suggests that this protooncogene may be important in this hematopoietic pathway. Since HL60 cells induced to differentiate to granulocytes do not express appreciable levels of c-fos transcripts (5), expression of this protooncogene in leukocytes in the absence of proliferation appears restricted to monocytes/macrophages.

We have examined the presence of c-fos-specific transcripts in freshly isolated human leukocyte populations. Unexpectedly, polymorphonuclear leukocytes (PMN) constitutively expressed high levels of c-fos mRNA. Monocytes expressed appreciable c-fos transcripts, though at much lower levels than PMN, and lymphoid cells showed no detectable expression. Exposure of PMN to agents that stimulate their function resulted in augmented levels of c-fos transcripts. Thus, expression of this protooncogene is not peculiar, among circulating leukocytes, to monocytes/macrophages.
to mononuclear phagocytes, and constitutive and inducible c-fos expression may play a role in the regulation of terminally differentiated circulating myeloid cells.

Materials and Methods

Cells. Leukocyte populations were purified as described in detail (9). Briefly, lymphocytes deprived of monocyte contamination (<1%) were obtained by adherence on plastic followed by passage through a nylon wool column. Monocytes (>95% pure, usually 97-98%) were purified using a one-step discontinuous Percoll (46%) (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient (9). PMN were isolated by dextran sedimentation (9). In selected experiments, PMN populations were further deprived of monocytes by treatment with a monocyte-specific mAb (UCHM1; reference 9, a kind gift of Dr. P. C. L. Beverly, University College, London, United Kingdom) and rabbit complement (Cederlane Laboratories Ltd., Hornby, Ontario, Canada). The final mononuclear contamination was <1%. As previously reported (10), the ratio of platelets to white cells was <0.5:1, as judged by phase-contrast light microscopy, and we were not able to detect any platelet contamination of PMN populations. The addition of platelet-enriched plasma (10%) to leukocytes or differentiated HL-60 cells did not affect the levels of c-fos expression.

All culture media and reagents were checked for endotoxin contamination by Limulus Amebocyte Assay (sensitivity 0.1-0.2 ng/ml; Microbiological Associates, Walkersville, MD) and found to be negative.

Stimuli. Human recombinant tumor necrosis factor (hrTNF; batch LYM12-10148B; sp act, $1.5 \times 10^7$ U/mg protein) was a kind gift of Dr. L. S. Lin (Cetus Corp., Emeryville, CA). Human recombinant granulocyte and granulocyte/macrophage colony-stimulating factor (hrGCSF and hrGMCSF, sp act $8 \times 10^7$ and $4 \times 10^7$ U/ml respectively) were obtained from Dr. L. Souza (AMGene, Thousands Oaks, CA) and Dr. S. Gillis (Immunex Corp., Seattle, Washington).

PMN in RPMI 1640 medium 10% FCS (Gibco, Glasgow, United Kingdom) ($5 \times 10^6$ cells/ml) were incubated with the various agents (1-20 h) at various concentrations. Cycloheximide (CH) and PMA were from Sigma Chemical Co., St. Louis, MO; inactivated streptococci (OK-432) were from Chugai Pharmaceutical Ltd., Tokyo, Japan. HL60 and U937 were induced to differentiate by culturing 3 x $10^6$ to $10^6$ cells/ml in complete medium for 1 h to 3 d in the presence of PMA (20 ng/ml) or (HL60 cells) DMSO (1.5% vol/vol).

Analysis of c-fos mRNA level. Total cellular RNA was isolated by guanidine isothiocyanate method (11). To avoid nucleic acid degradation by cytoplasmatic enzymes, in particular during isolation of RNA from PMN, the entire procedure was carried out in ice-cold conditions. 15 µg of RNA, unless otherwise stated, were analyzed by electrophoresis through 1% agarose formaldehyde gels, followed by Northern blot transfer to Gene Screen Plus membranes (New England Nuclear, Boston, MA). The plasmids pc-fos-3 (5) and α actin were labeled to high specific activity with α-[32P]dCTP (3,000 Ci/mmol; Radiochemical Center, Buckinghamshire, United Kingdom) by nick translation (11). Membranes were pretreated and hybridized in 50% formamide (Merck & Co., Rahway, NJ) with 10% dextran sulfate (Sigma Chemical Co.) and washed two times with 2× SSC (1× SSC: 0.15 M sodium chloride, 0.015 M sodium citrate) and 1% SDS (Merck & Co.) at 60°C for 30 min and finally two times with 0.1× SSC at room temperature for 30 min. The membranes were exposed for 12-48 h at ~80°C using intensifying screens. c-fos probe was removed from membranes according to manufacturer's instruction and then hybridized to α actin probe under the same experimental conditions.

Results

We examined the presence of c-fos transcripts in leukocyte populations obtained from normal healthy donors. As shown in Fig. 1, RNA from lymphoid cells carefully deprived of phagocytes (<1% contaminating monocytes) showed no detectable hybridization signal (lane 1). In contrast, freshly isolated monocytes, in the absence of deliberate stimulation, expressed appreciable levels of c-
Figure 1. Expression of c-fos protooncogene in myelomonocytic cell lines and human peripheral blood leukocytes. RNA samples (15 μg) were as follows: (lane 1) peripheral blood lymphocytes; (lane 2) uninduced U937 cells; (lane 3) TPA-induced (20 ng/ml, 1-h treatment) U937 cells; (lane 4) DMSO-induced (1.5% vol/vol, 3-d treatment) HL60 cells; (lane 5) peripheral blood monocytes; (lane 6) peripheral blood PMN.

fos-related transcripts (lane 5). These results extend to circulating human monocytes previous observations on c-fos expression in mononuclear phagocytes from mice (4, 8) or in vitro differentiated cell lines (4, 5). Moreover, as expected (5), HL60 (data not shown) and U937 (Fig. 1, lane 3) induced to differentiate to monocytes/macrophages by PMA treatment expressed c-fos transcripts. In contrast to our expectations based on previous reports (5) that were confirmed by us (Fig. 1, lane 4), on HL60 induced to differentiate to granulocytes, PMN showed high levels of c-fos transcripts, with a hybridization band of 2.2 kb (Fig. 1, lane 6). In addition to the major 2.2-kb band, a weak hybridization band of 2.0 kb was visible, presumably caused by r-fos transcripts (5). The faint band at ~3.5 kb signal has been suggested to represent an unprocessed transcript (5) (see also Fig. 2). The results presented in Fig. 1 were obtained with PMN preparations that contained <2% contamination with mononuclear cells. The high levels of c-fos-specific transcripts were also found (data not shown) when PMN were treated with an antimonocyte mAb (UCHM1) and complement (<1% contaminating mononuclear cells). This finding, together with the fact that PMN had considerably higher levels of c-fos transcripts than purified monocytes (Fig. 1), excludes that c-fos expression in PMN is related to contaminating monocytes.

c-fos transcripts have been shown to be superinduced by exposure to cycloheximide (5, 6). Superinduction by CH was also found in PMN (Fig. 2, A 4, B lanes 3–4).

It has been shown that exposure of murine macrophages to LPS, which activates these cells but does not induce proliferation, and other agents, such as PMA and CSF-1 (reference 8 and Introna, M., personal communication), results in induction of transcripts of c-fos protooncogene. This prompted us to examine whether stimuli that modulate a number of functions of PMN altered c-fos transcription. In addition to PMA and bacterial products (inactivated streptococci, OK-432), classical inducers of various PMN functions, we chose for these experiments GCSF and GMCSF and TNF, since these cytokines have recently been shown to regulate various granulocyte functions (12–14). These stimuli enhanced the levels of c-fos transcripts (Fig. 2). Densitometric analysis revealed a two- to sixfold increase (10 experiments) of the 2.2-kb hybridization band. No proliferation of the terminally differentiated peripheral blood PMN occurred
FIGURE 2. Expression of c-fos protooncogene in stimulated human peripheral blood PMN. A, B, and C represent three different experiments. RNA samples (15 μg except for A, lane 3, in which 6 μg of RNA were applied to the gel) were as follows: (A) (lane 1): uninduced U937 cells; (lane 2) PMA-induced U937 cells; (lane 3) peripheral blood PMN; (lane 4) cycloheximide-treated (10 μg/ml, 1 h) PMN; (lane 5) TNF-treated (400 U/ml, 1 h) PMN; (lane 6) G-CSF-treated (100 U/ml, 1 h) PMN; (lane 7) GM-CSF-treated (100 U/ml, 1 h) PMN. (B) (lane 1) Peripheral blood PMN; (lane 2) cycloheximide-stimulated (10 μg/ml, 1 h) PMN; (lane 3) PMA-stimulated (20 ng/ml, 1 h) PMN; (lane 4) PMN stimulated with PMA and cycloheximide; (lane 5) TNF-stimulated (400 U/ml, 1 h) PMN. (C) (lane 1) Peripheral blood PMN; (lane 2) inactivated streptococci (OK-432)-stimulated (0.1 mg/ml, 1 h) PMN.

after exposure to these agents, as assessed by [3H]TdT incorporation (results not shown).

Comparable levels of α actin transcripts were detected in various leukocyte populations and after PMN stimulation (not shown), thus excluding artifacts due to different transfer efficiency of RNAs or changes in total cellular mRNA.

The expression of c-fos gene was detectable also after 20 h of culture of PMN; the inducibility of c-fos was still detectable after 20 h of culture using PMA and TNF (data not shown).

Discussion

The results presented here demonstrate that freshly isolated PMN have high levels of c-fos transcripts. High c-fos expression in PMN is detectable in the absence of deliberate stimulation, after a separation carried out under Limulus-negative conditions. It is also noteworthy that monocytes had no procoagulant activity, a sensitive indicator of endotoxin contamination (10). Moreover, similar results were obtained when Polymixin B (1 μg/ml) was added throughout the separation procedure. It can therefore be operationally defined as constitutive. It is also noteworthy in this respect that c-fos transcripts in PMN were still detectable after 20 h of culture, whereas expression of this protooncogene associated with proliferation (1-2) and expression in macrophages augmented by LPS (8) are short-lived.

Expression of c-fos is associated with differentiation along the monocyte/macrophage lineage (4-8), and HL60-derived granulocytes do not express c-fos (5), an observation confirmed by us (Fig. 1, lane 4). The apparent discrepancy between results with HL60-derived granulocytes and freshly isolated PMN
is intriguing. The DMSO-driven differentiation of HL60 cells may not be representative of the in vivo conditions. CSF-1 has been shown to induce c-fos expression in murine macrophages (4), and it can be speculated that exposure to CSF during hematopoiesis is responsible for the presence of c-fos transcripts in PMN. In possible agreement with this hypothesis, in vitro exposure to GCSF and GMCSF enhanced c-fos expression in PMN.

PMN are terminally differentiated cells, incapable of proliferation, with minimal capacity to synthesize proteins and RNA and a definite lifespan in vitro and in vivo (15). However, various cytokines, such as TNF and CSF, potentiate various PMN functions (12–14). These cytokines, as well as classical stimuli of phagocytes (PMA and bacterial products), increase the levels of c-fos transcripts in PMN and this protooncogene may be involved in regulating the functional status of mature myelocytes.

In conclusion, unlike HL60-derived granulocytes, freshly isolated PMN show high levels of c-fos transcripts, and in the myelomonocytic differentiation pathway, expression of this protooncogene is not peculiar to commitment to the mononuclear phagocyte system. Terminally differentiated circulating mature PMN may represent a privileged system in which the possible link between protooncogene expression and stimulation of cell function, in the absence of proliferation, can be investigated.

Summary

We have investigated by Northern blot analysis the expression of c-fos protooncogene in human peripheral blood polymorphonuclear leukocytes (PMN). Freshly isolated PMN, unlike highly purified circulating lymphoid cells, showed high levels of c-fos transcripts. Appreciable c-fos mRNA was detected in monocytes, but in lesser amounts compared with PMN. Upon exposure to a series of agents that functionally stimulate granulocytes (PMA, inactivated streptococci, TNF, granulocyte and granulocyte/macrophage colony-stimulating factor), a considerable increase in c-fos transcripts was detected. Expression of c-fos in PMN was superinduced by exposure to cycloheximide. These data indicate that the myelomonocytic differentiation pathway c-fos expression is not peculiar to monocytes/macrophages and that PMN may represent a suitable system with which to investigate the link between protooncogene expression and functional activation.

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