Expression of c-jun Protooncogene in Human Myelomonocytic Cells

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A prototypic “immediate early” gene, c-fos, has been extensively investigated in relation to the differentiation and activation of myelomonocytic cells. The c-fos gene product is associated in transcriptional complexes with the c-jun product. These protooncogenes are part of the regulatory network of gene expression. The present study was designed to investigate expression of the c-jun protooncogene in human circulating myelomonocytic cells. We found that c-jun is constitutively expressed in normal monocytes and granulocytes, whereas low levels of transcripts are found in lymphocytes. Acute myelogenous leukemia (AML) samples of French–American–British Cooperative Group (FAB) subtypes 1 through 4 express appreciable levels of this protooncogene. Normal phytohemagglutinin (PHA)-activated lymphocytes express high levels of c-jun. Expression in normal myelomonocytic cells is detectable even after 18 hours of culture. The c-jun transcripts in myelomonocytic cells have a half-life of approximately 20 minutes and are superinduced by cycloheximide, which affects both the degradation rate of mRNA and the transcriptional activity of the c-jun gene. Functional activation of monocytes and granulocytes with phorbol esters, lipopolysaccharide, and tumor necrosis factor (TNF) increase c-jun expression. This induction is rapid, transient, and does not require intervening protein synthesis. Runoff experiments showed that in freshly isolated untreated monocytes, the c-jun gene is constitutively transcribed, and that induction by lipopolysaccharide is at least in part at the transcriptional level. Moreover, lipopolysaccharide (LPS) treatment reduced the degradation rate of c-jun transcripts, prolonging the half-life to approximately two hours. Expression of c-jun in resting and activated monocytes and granulocytes suggests that this protoonco gene may play a role in the differentiation and activation of cells belonging to the myelomonocytic lineage.

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MATERIALS AND METHODS

Cell culture reagents. The following reagents were used for culture and separation of cells: pyrogen-free saline (PBS) and distilled water for clinical use (Bieffe, Bergamo, Italy); RPMI 1640 medium (GIBCO, Glasgow, Scotland); glutamine (GIBCO); aseptically collected fetal calf serum (FCS; Hyclone, Steril System, Logan, Utah). The cell-culture medium routinely used was RPMI 1640 with 2 mmol/L glutamine and 10% FCS (complete medium). All reagents were tested for endotoxin contamination by Limulus

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Amebocyte lysate assay (Microbiological Associates, Walkersville, MD) with a sensitivity in our hands of 0.1 to 0.2 ng/mL of *Escherichia coli* Westphal lipopolysaccharide and found to be negative.

**Cells.** Leukocytes of heparanized fresh venous blood from healthy donors were purified as described elsewhere. Briefly, the mononuclear cell fraction was obtained by Ficoll density-gradient sedimentation (Ficoll–Trenlosung, Seromed–Biochem KG, Berlin, FRG) at the interface. Monocytes (≥95% pure, usually 97% to 98%) were purified using a one-step discontinuous Percoll gradient (46%) (Pharmacia Fine Chemicals, Uppsala, Sweden). Lymphocytes, which constitute the pellet of this Percoll gradient, were washed in PBS and further depleted of monocytes by plastic adherence. PMN were collected from the pellet of Ficoll gradient, washed in PBS and further depleted of monocytes by plastic adherence.

PMN were purified using a one-step discontinuous Percoll gradient sedimentation (Ficoll–Hypemnlosung, Senomed-Biochem KG, Berlin, FRG), and layered on top of 60% Percoll solution (285 mOsm), and layered on top of 60% Percoll solution (285 mOsm) in complete medium. Tubes were spun at 440 x g for 20 minutes at room temperature. The PMN-enriched fraction was recovered at the interface, washed twice with PBS, and resuspended in complete medium.

**Adherence.** PMN-ennriched fraction was incubated with myelomonocytic cells. Leukemic cell cultures, which constitute the pellet of this Percoll gradient, were washed in PBS and further depleted of monocytes by plastic adherence. PMN recovery was 90%, and red blood cell contamination was less than 5%.

The mononuclear cell fraction of circulating cells from patients with acute myelogenous leukemia (AML) was obtained by Ficoll–Hypaque. The diagnosis and classification of AML were made by standard criteria, according to morphology, cytochemical staining, and surface-marker analysis, using a panel of antibodies directed against antigens expressed by myelomonocytic cells. Leukemic cell preparations usually contained >90% blasts.

**Stimuli.** PMA, cycloheximide (CH), and actinomycin-D (ActD) were purchased from Sigma (St Louis). Lipopolysaccharide (LPS, *Salmonella enteritidis*) was from Difco Laboratories (Detroit). Recombinant tumor necrosis factor (TNF) was from BSF/Knoll (FRG). Purified phytomagglutinin (PHA) was from Welcome Research Laboratories (Beckenham, UK).

**Analysis of mRNA levels.** Northern blot analysis was carried out according to standard procedures. Total RNA was isolated by the guanidine isothiocyanate method, with minor modifications. Total RNA (15 μg) was analyzed by electrophoresis through 1% agarose formaldehyde gels, followed by Northern blot transfer to Gene Screen Plus sheets (New England Nuclear, Boston). The plasmid, containing a mouse cDNA c-jun clone (AH119), a kind gift from Dr R. Bravo, European Molecular Biology Laboratory, Heidelberg, FRG, was nick-translated with α-32PdCTP (3,000 Ci/mmol; Amersham, Uppsala, Sweden). The RNA was then precipitated by adding 880 μL ethanol. The pellet was resuspended in 100 μL hybridization solution (DES 10 mL/m, SDS 0.2%; EDTA 10mmol/L; NaCl 300 mmol/L), and radioactivity was checked with a β counter. RNA solution was hybridized at 65°C for 48 hours to DNA immobilized to nitrocellulose filters. In a given experiment, each filter was hybridized with the same number of cpm. The filters were then washed with several changes of 0.2x SSC at 65°C for 30 minutes and incubated at 37°C in 0.2x SSC with 1 μg/mL RNAse A for 30 minutes. Filter were then exposed for autoradiography as described above. For immobilization of DNA to filters, plasmids (5 μg) were denatured with 0.3 mol/L NaOH at 60°C for 30 minutes, neutralized with ammonium acetate (final concentration 4mol/L), and spotted onto a nitrocellulose filter (Schleicher and Schuell, Dassel, FRG) using a slot-blot apparatus (Schleicher and Schuell). Plasmids used contained a murine c-fos genomic clone (pc-fos-3) and a c-jun cDNA (clone AH119). As a negative hybridization control, an equal amount of pBR322 was transferred to the membranes.

**RESULTS**

Total RNA was extracted from highly purified leukocyte populations isolated from human peripheral blood and analyzed by Northern blot technique using a c-jun-specific probe. As shown in Fig 1, appreciable levels of c-jun mRNA are detectable in monocytes and granulocytes, whereas low levels were evident in lymphocytes. Since c-jun is induced by growth factors in fibroblast cell lines, we examined PHA-

![Fig 1. Expression of c-jun protooncogene in human circulating leukocytes. (A) Lane 1: PHA-activated (3 days, 1 μg/mL) lymphocytes. Lane 2: untreated lymphocytes. Lane 3: human circulating PMN. Lane 4: human circulating monocytes. (B) Lane 1: freshly isolated monocytes. Lane 2: monocytes cultivated for 18 hours at 37°C.](image-url)
activated lymphocytes (Fig 1) in which very high levels of c-jun transcripts were detectable after three days of mitogenic stimulation. The size of c-jun mRNA is 2.6 to 2.7 kb, as already reported. In some cases (see experiments below) a minor band of 3.4 kb, suggested to be a precursor molecule, was evident.

The "constitutive" c-jun expression of myelomonocytic cells is long-lasting in that it can be detected after cultivation of monocytes (Fig 1) and PMN (not shown) for 18 hours. No appreciable changes in mRNA levels were found when the same cellular population was analyzed immediately after separation or after 18 hours of culture (Fig 1).

To determine the half-life of c-jun mRNA in normal myelomonocytic cells, we blocked gene transcription by ActD, and then c-jun transcripts were analyzed at various time intervals. As shown in Fig 2, both in monocytes and PMN, the half-life of c-jun mRNA was approximately 20 minutes, as assessed by densitometric analysis.

Short-lived mRNAs are typically superinduced by exposure to protein-synthesis inhibitors. When monocytes and PMN were treated with CH for one hour, we found that c-jun expression was considerably superinduced (Fig 2). To investigate the mechanism by which the inhibition of protein synthesis leads to c-jun superinduction, we treated monocytes and PMN with CH and ActD. As shown in Fig 2, the half-life of mRNA in the presence of CH was prolonged to ≥ two hours; moreover, since CH induction in the presence of a transcriptional block was less than in the absence of ActD, we conclude that protein-synthesis inhibition acts also through an increase of c-jun gene transcription (see also runoff experiments below).

Previous data from our laboratory and others had shown that the c-fos protooncogene is rapidly induced in functionally myelomonocytic activated cells in the absence of detectable proliferation. Therefore, we investigated c-jun expression in nonproliferating, functionally activated, circulating myelomonocytic cells. Results (Fig 3) demonstrate that treatment of these cells with phorbol esters and LPS (for monocytes), or TNF (for PMN), for one hour enhanced the levels of c-jun transcripts. Densitometric analysis showed a twofold to fivefold increase of the 2.6 to 2.7-kb band. Under our conditions, no proliferation of terminally differentiated cells was observed.
monocytes and PMN occurred after exposure to these agents, as assessed by $^{3}$H TdR incorporation (data not shown). Induction of c-jun by LPS and PMA was further superinduced by the concomitant addition of CH, showing that gene induction does not require protein synthesis (Fig 3).

When the kinetics of c-jun induction by LPS in monocytes was examined, we found that maximal levels were detectable after one hour, whereas after four hours of treatment, c-jun transcripts fell to baseline levels (data not shown).

To investigate the mechanism by which c-jun is augmented in myelomonocytic cells, we carried out nuclear runoff experiments. Results, shown in Fig 4, demonstrate that the c-jun gene is actively transcribed in untreated monocytes, indicating that this gene is actually activated at the transcriptional level in freshly isolated resting monocytes. Thus, the constitutive expression of c-jun found in monocytes can be accounted for, at least in part, by transcription of the gene. Moreover, CH-and LPS-activated cells showed an augmented hybridization signal, demonstrating that these agents activate c-jun expression at the transcriptional level. When transcription of interleukin-1 and alfa-actin were tested in our runoff experiments, the former gene was found to be actively transcribed, whereas the levels of actin did not appreciably change (data not shown).

To investigate the possibility that activating agents can also affect the half-life of c-jun mRNA, circulating monocytes were stimulated with LPS in the presence of ActD. As shown in Fig 5, the levels of c-jun transcripts are considerably higher in cells treated with LPS and ActD compared with controls in which cells are treated with ActD only, with an estimated half-life of approximately two hours. Hence, LPS augments c-jun mRNA levels in monocytes by activating gene transcription and by prolonging half-life.

Having established that c-jun is expressed by myelomonocytic cells, it was of interest to obtain preliminary indications on expression of this protooncogene in AML. The functionally related immediate early gene c-fos has been found expressed in AML, with somewhat conflicting results as to the subtype specificity of expression. As shown in Fig 6, c-jun transcripts were detected in all seven cases examined representative of M1 through M4 AML. There were wide individual variations in transcript levels (see patient 4 v 5, both M3), with no obvious correlation with the AML subtype.

**DISCUSSION**

The aim of this study was to analyze the expression of the c-jun protooncogene in freshly isolated human peripheral-blood leukocytes. Our results indicate that among normal leukocytes, resting blood lymphocytes express barely detectable transcripts, whereas terminally differentiated cells of the myelomonocytic lineage show high levels of c-jun expression. As expected, mitogen-stimulated proliferating lymphocytes expressed high levels of c-jun mRNA.

Expression of c-jun in monocytes and PMN is detectable in the absence of deliberate stimulation, after a separation procedure carried out under Limulus-negative conditions. Moreover, under our experimental conditions, monocytes

Fig 5. Effects of LPS stimulation on the c-jun transcript half-life. RNA samples were as follows. Lane 1: LPS-treated (10 $\mu$g/mL, one hour) monocytes. Lane 2: untreated monocytes. Lane 3: monocytes treated with ActD (1 $\mu$g/mL, one hour). Lane 4: monocytes treated with ActD (1 $\mu$g/mL, two hours). Lane 5: monocytes treated with ActD and LPS (10 $\mu$g/mL) for one hour. Lane 6: monocytes treated with ActD and LPS for two hours.

Fig 4. Nuclear runoff analysis of c-jun transcription in human circulating monocytes treated with LPS or CH. (A) DNA samples hybridized to membranes were (a) c-fos, (b) c-jun, (c) pBR322. Transcripts were from untreated (lane 1) or LPS-treated (10 $\mu$g/mL, 30 minutes) monocytes. (B) DNA samples hybridized to membranes were (a) pBR322, (b) c-fos, (c) c-jun. Transcripts were from untreated (lane 1) or cycloheximide-treated (10 $\mu$g/mL, 30 minutes) monocytes.
had no procoagulant activity and did not produce interleukin-1 (data not shown), sensitive indicators of endotoxin contamination. Therefore, the expression of c-jun in freshly isolated myelomonocytic cells can be operationally defined as constitutive. It is also noteworthy in this respect that c-jun transcripts were still detectable after 18 hours of culture, whereas expression of immediate early genes induced by stimuli is typically short-lived (see below).

Available data on regulation of c-jun expression demonstrate that this gene is activated in response to proliferative stimuli. The main characteristics of mitogen-induced c-jun expression are that it is rapid, transient, and does not require protein synthesis, suggesting the inclusion of c-jun in the family of early-response genes. Data described herein demonstrate that c-jun expression is not restricted to cells engaged in active proliferation but is also present in terminally differentiated nonproliferating myelomonocytic cells.

We analyzed the regulation of c-jun transcript levels in unstimulated myelomonocytic cells. The half-life of c-jun mRNA in monocytes and PMN was approximately 20 minutes. The protein-synthesis inhibitor CH augmented c-jun transcript levels in PMN and monocytes. CH-superinduction of c-jun mRNA was caused by a prolongation of the half-life of the messenger (T1/2 ≤ 2 hours) and to augmented transcription of the gene, as assessed by nuclear runoff. Similarly, superinduction of c-fos by CH in murine macrophages has been attributed both to prolonged half-life and to enhanced transcription. These two mechanisms of c-jun regulation are operative in serum-stimulated 3T3 fibroblasts.

Although early response genes have been implicated in controlling cell proliferation, recent data suggest that these genes are regulated in functionally activated myelomonocytic cells in the absence of proliferation. Myelomonocytic cells respond to exogenous and endogenous signals by reprogramming their functional status. Functional activation implies expression of genes whose products mediate a new set of functions. As the c-jun protooncogene belongs to early-response genes and encodes for a transcriptional activator, we examined the possibility that this gene could be modulated in functionally activated, nonproliferating myelomonocytic cells. We treated monocytes and PMN with the phorbol esters LPS and TNF, which are known to activate these cells for a series of functional parameters. Expression of c-jun was augmented under these experimental conditions with a kinetics that was rapid and transient. Moreover, our data indicate that LPS induction is at least in part at the transcriptional level and, for mitogen-induced c-jun, does not require intervening protein synthesis. LPS stimulation not only affects the transcriptional level but is also able to increase the half-life of c-jun mRNA.

The results described so far demonstrate that expression of c-jun is associated with myelomonocytic differentiation and activation in a way reminiscent of the functionally related c-fos gene. The latter gene has also been studied in human hematopoietic malignancies, with somewhat conflicting results. Mavilio et al and Pinto et al reported that c-fos was only or predominantly expressed in M4 and M5 cases, whereas Sariban et al and Preisler et al found expression in the less differentiated M1 and M2 cases. It was therefore of interest to obtain preliminary indications as to the expression of c-jun in AML. In the limited caselist examined in this study, c-jun expression was detected in M1 through M4 cases, suggesting that expression of this immediate early gene is not restricted to any specific AML subtype.

In conclusion, our data demonstrate that the c-jun protooncogene is expressed in freshly isolated myelomonocytic cells from human peripheral blood and that this expression is augmented in functionally activated cells even in the absence of proliferation. Thus, the pattern of expression of c-jun in normal human leukocytes parallels c-fos expression in these cells in many respects. Since the FOS protein has been shown to be associated with JUN/AP-1 to form a transcriptional activator, data presented here support the hypothesis that these two protooncogenes may play a role in regulating gene expression related on the one hand to myelomonocytic differentiation and on the other to the acquisition of a new set of functions induced by functional activation.

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