Regulation of the production of granulocyte-macrophage colony-stimulating factor by macrophage-like tumour cell lines

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Macrophage tumour cell lines (PU5-1.8, P388D1) produced detectable granulocyte macrophage colony-stimulating (CSF-2) activity measured using a factor-dependent cell line FDC-P1. The production of CSF-2 was enhanced by endotoxin and inhibited by serum, and correlated inversely with [^{3}H]TdR incorporation. mRNA isolated from PU5-1.8 or P388D1 cells initiated CSF-2 production when injected into *Xenopus laevis* oocytes. The specific activity in this assay was unaltered in mRNA isolated from endotoxin-treated cells. The results suggest that endotoxin acts at a post-transcriptional level.

CSF-2 *Endotoxin* *Tumor cell* *Regulation*

1. INTRODUCTION

Granulocyte-macrophage colony-stimulating factor (CSF-2) is a glycoprotein hormone that regulates colony formation by immature haemopoietic stem cells [1]. In addition, CSF-2 causes proliferation of mature macrophages [2,3]. Ralph et al. [4] have reported that macrophage-like tumour cells can be stimulated, by bacterial endotoxin, to release a granulocyte-macrophage colony-stimulating activity. Here, we have re-examined the production of CSF-2 activity by the macrophage-like tumour cell lines P388D1 and PU5-1.8 using, as an assay, the factor-dependent cell line FDC-P1 [5,6]. Our results suggest that P388D1 and PU5-1.8 are constitutive producers of CSF-2 and that induction by endotoxin does not involve an increase in CSF-2 specific mRNA.

2. MATERIALS AND METHODS

2.1. *Culture media*

PU5-1.8 cells were maintained in Dulbecco's modified Eagle's minimal essential medium, WEH1-3, P388D1 and FDC-P1 cells in RPM1-1640. All media contained 10% foetal bovine serum, 2 mM glutamine and gentamycin (60 U/ml). FDC-P1 cells additionally required 30% WEH1-3 conditioned medium as a source of interleukin 3 [5,6]. Endotoxin from the heptose-deficient mutant R595 of *Salmonella minnesota* (a gift from Professor W. Doe) was prepared, sonicated and dissolved in 0.1% triethylamine as in [7].

2.2. *RNA preparation*

RNA was prepared from batches of 10^9 WEH1-3, PU5-1.8 or P388D1 cells that were growing exponentially with or without 24 h pretreatment with 1 μg/ml endotoxin. RNA was extracted using guanidine isothyanate [8], and fractionated by centrifugation through a caesium chloride step gradient [9]. There was no difference in RNA yield from control or endotoxin-treated cells, 10^9 cells yielding 2–4 mg RNA. Poly(A)$^+$ RNA was isolated by one passage over an oligo(dT) cellulose column [10]. The biological activity of the poly(A)$^+$ RNA was checked in a rabbit reticulocyte lysate in vitro translation system [11] and no differences in specific activity per μg were found between preparations from endotoxin-treated and untreated cells.

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2.3. *Oocyte translates*

Fifty nl poly(A)$^+$ RNA (1 mg/ml in 10 mM Tris–HCl, pH 7.5, 1 mM EDTA) was injected into each of 15–18 *Xenopus laevis* oocytes. The oocytes were incubated for 48 h at 21°C in 100 μl medium [12]. Supernatants were harvested and assayed for FDC-P1 growth factor activity.

2.4. *Growth assays*

FDC-P1 cells were washed thoroughly and plated at 2×10^4 cells/well in 200 μl RPM1-1640 + 10% foetal bovine serum. The test samples were assayed in serial 1/2 dilutions starting from 1/4 final concentration. After 30 h, the cells were pulsed overnight (16 h) with 0.5 μCi [^{3}H]thymidine (TdR) (2 Ci/mmol; Amersham, Bucks) harvested automatically onto glass-fibre discs, and counted by liquid scintillation counting. ED_{50} units are defined as the amount of factor required to cause 1/2 maximal stimulation of [^{3}H]TdR incorporation. The assay detects approx. 0.005 ED_{50} units/ml. Proliferation of P388D1 and PU5-1.8 cells is assayed in the same way, except that the plates were subjected to a cycle of freeze-thawing to disrupt adherent cells before harvesting.

3. RESULTS AND DISCUSSION

FDC-P1 is a myelomonocytic line that requires either interleukin 3 (IL3) or CSF-2 for continued proliferation [6]. Table 1 shows a comparison of the rates of growth and release of FDC-P1 growth factor activity by P388D1 and PU5-1.8. In preliminary experiments, we established that 1 μg/ml endotoxin does not influence the titre of CSF-2 or IL3 activity measured on FDC-P1 cells (not shown). Like Ralph et al. [4], we observed that bacterial endotoxin inhibits the growth of PU5-1.8. In this respect, the cell lines differ from mouse bone marrow-derived macrophages [13] which are unaffected by 10-fold higher concentrations of endotoxin. P388D1 cells, which are poorly adherent, and proliferate faster than PU5-1.8, were less affected by endotoxin. Ralph et al. [4] could not detect constitutive secretion of CSF-2 using the conventional colony assay. However, the FDC-P1 assay is sufficiently sensitive to detect a low level of CSF-2 production even without added endotoxin. The concentration of serum is an important variable, and CSF-2 elaboration is maximal at between 0.5 and 1% serum. Endotoxin (1 μg/ml) causes an 8–10-fold enhancement of CSF-2 elaboration by PU5-1.8 cells at low serum concentrations. P388D1 cells, in addition to responding less strongly to the growth inhibitory effect of endotoxin, produce less CSF-2. Having established optimal conditions for production, we showed that none of the FDC-P1 growth factor activity in PU5-1.8 (or P388D1) conditioned medium could be attributed to IL3 since no activity was detectable using another factor-dependent cell line, 32D-C123, which responds to IL3 and interleukin 2, but not CSF-2 [6] (not shown). The absence of IL3 mRNA from P388D1 and PU5-1.8 cells has been confirmed using a specific cDNA probe [12] (KMS, DAH, in preparation).

We wished to ascertain whether the action of endotoxin was due to increased transcription of mRNA for CSF-2. To test this, we prepared

Table 1

Growth and production of CSF-2 by macrophage-like tumour cell lines

Serum	[^{3}H]TdR incorporation (cpm)		FDCP-1 growth factor	
PU5-1.8	–	+	–	+
0	8286	750	0.2	2.7
0.5	–	–	0.6	4.9
1	16045	5031	0.4	4.2
2	20316	9843	0.3	2.1
5	23375	13514	0.2	1.2
10	42691	14882	–	–
20	39365	23269	0.1	0.5
P388D1	–	+	1	+
0	19391	12193	0.1	0.3
0.5	38731	21438	0.5	1.3
1	55568	35631	0.5	1.2
2	56633	47099	0.5	1.0
5	52540	38193	0.4	1.0
10	61734	44454	–	–
20	65425	42458	0.3	0.5

FDC-P1 growth factor was assayed (section 2) in the supernatants of cells cultured 48 h at an initial density of 2×10^5/ml in 1 ml RPM1-1640 + appropriate additions of serum + or – endotoxin (1 μg/ml). Results are expressed as ED_{50} units/ml. Results are the average of duplicates. [^{3}H]TdR incorporation was assayed as described in section 2

poly(A)$^+$ RNA from control and endotoxin-stimulated PU5-1.8 and P388D1 cells. The yields of RNA and the specific activity in the rabbit reticulocyte lysate in vitro translation system were unaltered by the endotoxin treatment (section 2). Table 2 shows the results when this mRNA was injected into *X. laevis* oocytes and the supernatants assayed for CSF-2 activity.

In 3 separate experiments, we found no evidence for induction of translatable mRNA for CSF-2 by endotoxin. The amount of FDC-P1 growth factor produced by the oocytes injected with mRNA from P388D1 or PU5-1.8 was 30–40 times less than observed with WEH1-3 mRNA. This is not surprising, since, under comparable conditions, WEH1-3 cells produce up to 1000 times more FDC-P1 growth factor activity than endotoxin-stimulated PU5-1.8 cells being constitutive high-level secretors of IL3 [12]. The enhanced expression of IL3 mRNA in WEH1-3 cells permitted the recent cloning of this factor using the oocyte translate/FDC-P1 cell assay system [12].

The results suggest that macrophage-like tumour cell lines are constitutive producers of a growth factor (CSF-2) to which normal macrophages can respond [3]. This provides a possible explanation for their autonomous growth which contrasts with the factor-dependence of normal macrophages [2,13,14]. There are several other growth factors implicated in autocrine stimulation of growth, e.g., platelet-derived growth factor [15] and interleukin 2 [16], and tumourogenesis can result from transmission of an oncogene encoding an analogue of such a growth factor (see [15]). It will be interesting to see whether the recently published cDNA sequence for CSF-2 [17] resembles any oncogene.

Table 2

Translation of CSF-2 messenger RNA in *X. laevis* oocytes

mRNA source	FDC-P1 growth factor (ED_{50} units/ml)
WEH1-3B	7.3
PU5-1.8	0.23
PU5-1.8 (endotoxin-treated)	0.22
P388D1	0.20
P388D1 (endotoxin-treated)	0.21
Blank (buffer-injected)	0.005

The experiment was carried out as described in section 2. The limit of detection is 0.005 ED_{50} units/ml. The results are representative of 3 determinations using separate preparations of RNA and oocytes

Definite analysis of the mechanism of action of endotoxin on CSF-2 production will await the availability of specific assays for the receptor. However, we consider the most likely explanation to be an interference with the action of endogenous CSF-2, so that the factor no longer interacts with receptors on the producing cell, but is released into the medium. In support of this proposal, endotoxin has been shown to cause down regulation of CSF-1 receptors on mouse macrophages [18]. Further studies will be directed towards understanding interaction between endotoxin and receptors for CSF-2 on proliferating macrophages.

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