Differential control of proto-oncogene *c-myc* and *c-fos* expression in lymphocytes and fibroblasts

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In lymphocytes stimulated with the mitogen phytohaemagglutinin, an inhibitor of the enzyme ADP-ribosyltransferase (ADPRT) completely blocks the proliferative response and the increase in expression of the proto-oncogene *c-myc* without affecting *c-fos* significantly. Conversely, in fibroblasts the serum-induced growth is not affected by the ADPRT inhibitor, and both oncogenes are dramatically super-induced. Hence there are differences between lymphocyte and fibroblast early responses to mitogenic stimulation and also between regulation of *c-fos* and *c-myc* gene expression.

INTRODUCTION

The expression of *c-myc* and *c-fos* proto-oncogenes increases sharply soon after stimulation of various quiescent cell types, including fibroblasts and lymphocytes (Kelly *et al.*, 1983; Greenberg & Ziff, 1984; Kruijer *et al.*, 1984; Müller *et al.*, 1984; Reed *et al.*, 1986; Granelli-Piperno *et al.*, 1986; Moore *et al.*, 1986). The *c-fos* gene is induced more rapidly and is more transient than *c-myc*, although even the latter declines before the onset of DNA synthesis and does not appear to be regulated by the cell cycle (Thompson *et al.*, 1985; Hann *et al.*, 1985). These data have been used to support the notion of a common pathway for stimulating proliferation which is shared at least in part by lymphocytes and fibroblasts (Kelly *et al.*, 1983; Metcalfe *et al.*, 1985).

Inhibitors of the nuclear enzyme ADP-ribosyltransferase (ADPRT) block differentiation of several eukaryotic cells, including an early event in lymphocyte activation, without affecting proliferation *per se* (Farzaneh *et al.*, 1982; Johnstone & Williams, 1982; Althaus *et al.*, 1982; Greer & Kaplan, 1983; Williams & Johnstone, 1983). Here we report the effect of one ADPRT inhibitor, 3-methoxybenzamide, on the induction of *c-fos* and *c-myc* genes in both mitogen-stimulated human peripheral-blood lymphocytes and serum-stimulated fibroblasts. The results highlight some major early event differences between the two cell types.

EXPERIMENTAL

Cell culture

Human peripheral-blood lymphocytes (Johnstone, 1984) were cultured at 10^6 /ml in RPMI 1640 containing 10% (v/v) foetal-calf serum and PHA (Pharmacia; 2.5 µg/ml). For proliferation assays, flat-bottomed microtitre plates were used and [3 H]thymidine was added to a final concentration of 2 µCi/ml for 4 h before collecting the cells at 48 h with a cell harvester and determining the radioactivity incorporated into DNA by

liquid-scintillation counting. Growth of fibroblasts was studied by plating Swiss 3T3 cells (Flow) into 35 mm dishes (8×10^4 cells/dish) in RPMI medium containing 10% foetal-calf serum. When the cells had reached the desired density (usually after 3 days), the medium was removed and replaced with fresh medium containing 0.5% serum. When growth had ceased (1–2 days), the cells were restimulated with fresh medium containing 20% serum. Cell growth was assessed throughout by a protein dye-binding assay (Winterbourne, 1986). For some cultures of both lymphocytes and fibroblasts, the ADPRT inhibitor 3-methoxybenzamide was dissolved by vigorous stirring and gentle heating in RPMI to 20 mM and added to the cells to give the final concentration indicated for each experiment.

mRNA analysis by Northern blotting

The probes used were derived from the mouse genome and consisted of: for *c-myc*, the 1.4 kb restriction-*SacI* fragment containing the first protein coding exon (v5') (Neuberger & Calabi, 1983); for *c-fos*, the 3.4 kb endonuclease-*EcoRI*/*BamHI* fragment containing three of the four exons (Van Beveren *et al.*, 1983). Cells were cultured as described above, but scaled up to 75 cm² flasks for lymphocytes and 9 cm dishes for fibroblasts; proliferation was always also monitored in parallel cultures. After various times the cells were washed once with medium and RNA extracted by the guanidine isothiocyanate/CsCl method (Chirgwin *et al.*, 1979). A 10 µg portion of total RNA (quantified by A_{260}) was separated by electrophoresis in formaldehyde on a 1.5% agarose gel (Meinkoth & Wahl, 1984) and transferred to a Biotrans A membrane (Pall Process Filtration, Portsmouth, U.K.) according to the manufacturer's instructions. The membranes were prehybridized for 2 h, hybridized for 20 h with 32 P-labelled probe [Feinberg & Vogelstein, 1983; (1–2) $\times 10^8$ c.p.m./µg; approx. 10^7 c.p.m./ml] and then washed, all as described in the manufacturer's instructions. Autoradiography was carried out with a Cronex Lightning Plus screen

Abbreviations used: ADPRT, ADP-ribosyltransferase (EC 2.4.2.30); PHA, *Phaseolus vulgaris* (French bean) phytohaemagglutinin; kb, kilobase(s).

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Fig. 1. Effect of the ADPRT inhibitor 3-methoxybenzamide on mitogenic stimulation of lymphocytes and fibroblasts

(a) Lymphocytes were cultured with the indicated final concentration of 3-methoxybenzamide, added either 30 min before PHA (●) or 24 h after PHA (○), and the incorporation of [3 H]thymidine into DNA was determined at 48 h. The results (means $\pm$ s.d. for three independent experiments, each in triplicate) are expressed relative to cultures without 3-methoxybenzamide (200 000–400 000 c.p.m./ 10^6 cells); no adjustment was made for background incorporation without mitogen, which represented 2–5% of maximum. (b) Fibroblasts were plated at zero time and allowed to grow in 10% serum until 70 h (first arrow), when the medium was changed to 0.5% serum. When growth had ceased, after 99 h (second arrow), the medium was removed and replaced with fresh medium containing 0.5% serum (□) or 20% serum (■) or 20% serum plus 3 mM-3-methoxybenzamide (◇). Cell density, assessed by a protein dye-binding assay, is also presented (three 35 mm dishes for each point); the errors are encompassed by the symbols.

(du Pont) and Fuji RX film at -70°C . The densities of bands within an autoradiograph were compared by using a LKB Ultrascan laser densitometer.

RESULTS AND DISCUSSION

Competitive inhibitors of ADPRT, such as 3-amino- and 3-methoxy-benzamide (Purnell & Whish, 1980), when added slightly before, or at the same time as, mitogen, decreased the response of lymphocytes in a dose-dependent manner, such that 3 mM-3-methoxybenzamide caused 90–95% inhibition (Fig. 1a). This dose had no effect on the proliferation of lymphoblastoid and other cell lines (Althaus *et al.*, 1982; Farzaneh *et al.*, 1982; Greer & Kaplan, 1983; Williams, 1984; Johnstone, 1984), neither did it affect lymphocyte stimulation if added 24 h after mitogen, well before DNA synthesis commences (Fig. 1a), demonstrating that only an early event(s) in activation was susceptible to these chemicals (Johnstone & Williams, 1982; Johnstone, 1984). In contrast with lymphocyte activation, 3-methoxybenzamide had no effect on the serum-induced growth stimulation of quiescent fibroblasts (Fig. 1b), suggesting some fundamental differences between the two cell systems (Johnstone & Darling, 1985). ADPRT appears to be involved in differentiation, but not proliferation or its control (Williams & Johnstone, 1983; Hunting *et al.*, 1985).

Because of this difference between lymphocyte and fibroblast stimulation, although increased expression of

c-fos and *c-myc* has been implicated in both, we investigated the effect of the ADPRT inhibitor 3-methoxybenzamide (at a fixed dose of 3 mM throughout the study) on the oncogene expression in these two systems by using Northern blotting. Quantification of the autoradiographs by densitometry showed that PHA caused an increase of 20–100-fold (range of four determinations; the low levels without stimulation make exact comparisons difficult) in the amount of *c-myc* mRNA in human peripheral lymphocytes. The increase was detectable from about 1 h after stimulation and was maximal at 4–12 h (full data not shown), in agreement with earlier studies using this system (Reed *et al.*, 1986) and with those on other species and with other mitogens (Kelly *et al.*, 1983; Granelli-Piperno *et al.*, 1986; Moore *et al.*, 1986). Under the same conditions, *c-fos* mRNA peaked at 45–60 min and decreased to background by 2–3 h (full data not shown), somewhat more slowly than was the case with other species or mitogens (Granelli-Piperno *et al.*, 1986; Moore *et al.*, 1986), but agreeing with an earlier study using this system (Reed *et al.*, 1986). 3-Methoxybenzamide added 30 min before PHA blocked the increased *c-myc* expression (90–99% inhibition from three densitometric experiments; an example is shown in Fig. 2a), but had no significant effect on *c-fos* (an example is shown in Fig. 3a). ADPRT inhibitors added to lymphocytes slightly before mitogen stimulation prevent the subsequent increases in protein and DNA synthesis, but do not affect some very early membrane-related events such as mitogen binding, increased

Fig. 2. Northern-blot analysis of the expression of *c-myc* in mitogen-stimulated lymphocytes and fibroblasts

(a) Lymphocytes were stimulated with PHA for the times indicated; (b) fibroblasts, made quiescent by serum deprivation as in Fig. 1(b), were either left without serum or stimulated with 20% serum for 1 h. 3-Methoxybenzamide (3 mb; 3 mM) was added to some cultures, as indicated, 30 min before the mitogen. Total RNA was extracted, and the level of *c-myc* mRNA was investigated by Northern blotting. The migration positions of 18 S and 28 S ribosomal RNA on the gels are indicated by the arrows.

Table 1. Super-induction of *c-myc* and *c-fos* in serum-stimulated fibroblasts by 3-methoxybenzamide

The expression of the proto-oncogenes was quantified by densitometry of the autoradiography band from Northern blots in six independent experiments (see the Experimental section for further details). The absorbance is presented in arbitrary units, the super-induction being calculated as a ratio of the serum-plus-3-methoxybenzamide (3 mb) value to the serum alone value. In most cases without serum stimulation, especially for *c-myc*, the bands are too faint for meaningful quantification (indicated by *).

Expt. no.	Oncogene detected	Time after stimulation	Absorbance of autoradiography band				Super-induction ratio
			No additions	3 mb	Serum	Serum + 3 mb	
1	<i>c-myc</i>	1 h	*	*	1125	3398	3.0
2	<i>c-myc</i>	1 h	*	*	3302	8732	2.6
3	<i>c-myc</i>	2 h	*	*	250	2370	9.5
4	<i>c-fos</i>	30 min	286	226	871	1716	2.0
5	<i>c-fos</i>	30 min	*	*	266	6483	24.4
6	<i>c-fos</i>	1 h	514	738	2873	17074	5.9

turnover of phosphatidylinositol (Johnstone, 1984) and increases in membrane chemiluminescence and cytoplasmic Ca^{2+} concentration (A. J. H. Gearing, A. P. Johnstone, S. L. King, K. Meréty & G. St. J. Whitley, unpublished work). The present data further delineate the requirement for ADPRT for increased *c-myc*, but not *c-fos*, expression. 3-Methoxybenzamide is the only chemical, other than 8-bromo cyclic AMP (Moore *et al.*, 1986), yet reported to block the *c-myc* increase completely; cyclosporin, dexamethasone and T11A antibody give only partial inhibition (Reed *et al.*, 1986; Granelli-Piperno *et al.*, 1986).

In contrast with lymphocytes, 3-methoxybenzamide did not inhibit *c-myc* induction in fibroblasts; in fact it caused a dramatic super-induction of both oncogenes. Details of six experiments at various times of stimulation are presented in Table 1, and one example for each oncogene is shown in Figs. 2(b) and 3(b). The inhibitor had no effect on the oncogene expression of quiescent

fibroblasts, but acted synergistically with serum to cause a 9.0 ± 4.0 -fold enhancement over that caused by serum alone (mean $\pm$ S.E.M. for six experiments): a greater effect than that of other super-inducing agents, such as cycloheximide (Kelly *et al.*, 1983; Kruijer *et al.*, 1984; Müller *et al.*, 1984). The super-induction of both oncogenes did not increase the growth rates of the fibroblasts (Fig. 1b). The effect of cycloheximide has been interpreted as inhibition of the synthesis of a labile protein that either represses transcription or increases degradation of the oncogene mRNA, presumably specifically. The benzamide derivatives do not inhibit protein synthesis (Purnell & Whish, 1980; Johnstone, 1984; Hunting *et al.*, 1985), as exemplified in Fig. 1. They do, however, inhibit differentiation, consistent with their blocking new gene expression (Farzaneh *et al.*, 1982; Johnstone & Williams, 1982; Althaus *et al.*, 1982; Greer & Kaplan, 1983; Williams & Johnstone, 1983). Hence the simplest explanation of the super-induction would

Fig. 3. Northern blot analysis of the expression of *c-fos* in mitogen-stimulated lymphocytes and fibroblasts

Details are as described for Fig. 2, except that the fibroblast culture time was 30 min.

appear to be that they are blocking the induction of the genes coding for the labile controlling proteins.

The increase in levels of *c-fos* and *c-myc* mRNA after stimulation of fibroblasts appears to involve both increased transcription and decreased mRNA degradation (Greenberg & Ziff, 1984; Blanchard *et al.*, 1985). The situation in lymphocytes has not been studied so well, but for *c-myc* it involves at least a contribution from increased transcription (Granelli-Piperno *et al.*, 1986). The present observation, that 3-methoxybenzamide neither inhibits nor super-induces *c-fos* expression in mitogen-stimulated lymphocytes, implies a control mechanism different from that in fibroblasts. This, together with the relatively high level of *c-fos* expression in unprimed quiescent human lymphocytes (Fig. 3a and Granelli-Piperno *et al.*, 1986) would support the view that *c-fos* serves different functions in different cell types (Müller *et al.*, 1985).

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