Vascular smooth muscle (VSM) cell proliferation contributes to the pathogenesis of atherosclerosis, restenosis after angioplasty and vein graft disease. The regulation of genes involved in VSM cell proliferation, particularly by naturally occurring inhibitors, is therefore of some importance. We have investigated the role of the c-myc proto-oncogene in growth arrest of exponentially proliferating rat VSM cells, following mitogen withdrawal, treatment with heparin (50 μg/ml), interferon-γ (IFN-γ) (100 i.u./ml), or the cyclic nucleotide analogues, 8-bromo-adenosine-3’5’-cyclic monophosphate (8-Br-cAMP; 0.1 mM) and 8-bromoguanosine-3’5’-cyclic monophosphate (8-Br-cGMP; 0.1 mM). Growth arrest was accompanied by down-regulation of c-Myc protein and mRNA following treatment with all inhibitors. Serum withdrawal or IFN-γ treatment suppressed c-myc expression by more than 50% within 2 h, and this occurred throughout the cell cycle. Platelet-derived growth factor, epidermal growth factor and basic fibroblast growth factor all contributed independently to the maintenance of c-myc expression. Heparin, 8-Br-cAMP or 8-Br-cGMP also suppressed c-myc, but this occurred later, after 24–48 h, and was also observed following arrest by metabolic block. We conclude that c-myc expression is linked to VSM cell growth arrest in response to endogenous regulators and metabolic block. Down-regulation of c-myc expression may thus be an essential part of the arrest programme in VSM cells induced by many pharmacological agents.

INTRODUCTION

Vascular smooth muscle (VSM) cell accumulation is an important component of the pathology of atherosclerosis (Davies, 1987). The VSM cells present in these lesions are thought to have arisen by both migration of cells from the media and proliferation (Ross, 1993), albeit at low levels (Gordon et al., 1990; O’Brien et al., 1993) over long periods or in an episodic fashion. It is not known whether this reflects excess proliferation in response to specific mitogens, or failure to arrest in response to growth inhibitors, or a combination of both. There is, therefore, considerable interest in the mechanism by which proliferating VSM cells arrest in response to naturally occurring agents and their pharmacological analogues.

Growth arrest of VSM cells may involve down-regulation of genes required to maintain proliferation, among which the c-myc proto-oncogene appears to be important. The c-Myc protein is a short-lived, nuclear phosphoprotein which binds DNA in a sequence-specific manner and possesses direct transcriptional activity (Blackwell et al., 1990; Kato et al., 1990). Deregulated expression of c-myc is very frequent in human tumour cells (Spencer and Groudine, 1991), and is sufficient alone to drive cell proliferation, even in the absence of mitogens (Eilers et al., 1989; Evan et al., 1992), to inhibit differentiation (Coppola and Cole, 1986; Prochownik and Kukowska, 1986), and to initiate programmed cell death (apoptosis) (Evan et al., 1992; Bennett et al., 1994a). In quiescent VSM cells, c-myc mRNA levels rise rapidly after mitogen stimulation (Kindy and Sonenshein, 1986; Gadeau et al., 1991) as cells enter the cell cycle. However, unlike many other immediate early genes, c-myc expression is then maintained at a constant level throughout the cell cycle providing that mitogens are present (Gadeau et al., 1991; Campan et al., 1992). This steady-state expression may thus be involved in maintaining VSM cell proliferation and preventing growth arrest. Indeed, we have recently shown that deregulated expression of c-myc at low levels can promote VSM cell proliferation, and induce partial dedifferentiation and apoptosis (Bennett et al., 1994a). Furthermore, antisense oligonucleotides to c-myc can suppress VSM cell proliferation in both animal and human cells (Ebbecke et al., 1992; Biro et al., 1993; Bennett et al., 1994b). Thus down-regulation of c-myc expression from these low steady-state levels may be an important part of the growth arrest process in VSM cells.

Proliferation of VSM cells in culture can be inhibited by a variety of agents, including heparin, interferon (IFN), elevation of intracellular cyclic nucleotide levels, (cyclic AMP and cyclic GMP), transforming growth factor β (TGF-β) and serum deprivation. A number of studies have investigated the effect of these agents on entry into S-phase and c-myc expression in quiescent VSM cells. IFN inhibits S-phase entry in fibroblasts and VSM cells re-stimulated with serum (Einat et al., 1985b; Heyns et al., 1985; Fukumoto et al., 1988), and decreases expression of c-myc (Einat et al., 1985a). Heparin also reduces S-phase entry in serum-stimulated quiescent VSM cells, but the effect on c-myc expression is more controversial. Wright et al. (1989) and Pukac et al. (1990, 1992) reported that heparin reduces c-myc expression, whereas Reilly et al. (1989) found no effect. Suppression of c-myc expression using antisense oligonucleotides can directly suppress cell cycle entry but also arrest proliferating cells (Bennett et al., 1994b), indicating a role for c-myc not only on the G0/G1
transition, but also later in the cell cycle. This implies that suppression of c-myc expression may be part of a pathway of growth arrest shared by a number of inhibitors. However, while effects on G_0/G_1, cells have been well documented, the inhibitory effect of agents on VSM cells that are already proliferating does not appear to have been studied. Studies from other cell lines show that regulation of c-myc expression is dependent on cell-cycle phase and cell density (Dean et al., 1986; Waters et al., 1991) and evidence from studies examining serum-stimulated quiescent cells cannot be extrapolated to proliferating cells. We have therefore examined the effect of several naturally occurring growth inhibitors or their analogues on growth arrest and c-myc expression in proliferating VSM cells.

MATERIALS AND METHODS

Cell culture

Thoracic aortae from 6-week-old Sprague-Dawley rats were removed, cleaned of fat and adherent connective tissue, and medial strips lifted from the underlying adventitia. The strips were divided into approx. 1 mm² pieces by crossed scalpels and the explants cast into 48-well plates (Falcon) coated with 0.1 % type-1 collagen (Sigma). Cells were grown in Dulbecco’s modified essential medium (DMEM) containing 10 % fetal calf serum (FCS), 20 mM Hepes (Flow), and equilibrated with 95 % air and 5 % CO_2. Subconfluent cell cultures were passaged by trypsinization in 0.05 % trypsin in PBS and re-seeded in complete growth medium.

Experiments were conducted using cells between passages 8 and 14.

Assessment of cell number and [³H]thymidine incorporation

Cells (1 × 10⁶) were plated into the wells of a 12-well plate (Falcon) and grown in DMEM containing 10 % (v/v) FCS for 48 h. The cells were then washed three times in PBS and the medium substituted with DMEM containing 0.5 % FCS, 10 % FCS, or 10 % FCS supplemented with 50 μg/ml of sodium heparin type-1 (Sigma), 0.1 mM 8-bromoadenosine-3’5’-cyclic monophosphate (8-Br-cAMP) (Sigma), 0.1 mM 8-bromo-guanosine-3’5’-cyclic monophosphate (8-Br-cGMP) (Sigma) or 100 i.u./ml of recombinant rat IFN-γ as appropriate. These concentrations of pharmacological agents were chosen following preliminary experiments which suggested that they result in approx. 50 % inhibition of proliferation (ID₅₀) of exponentially dividing rat VSM cells after 48 h. Cells were labelled with 1 μCi/ml of [³H]thymidine (Amersham) for 18 h and harvested by trypsinization from triplicate wells at 1, 3, 6 or 8 days. Live cell numbers were determined by Trypan Blue exclusion using a haemocytometer. Cells from duplicate wells were washed three times in cold PBS, pelleted and lysed in 0.5 ml of 1 % SDS, and incubated on ice for 5 min. PBS (1 ml) and 15 % (v/v) trichloroacetic acid (1.5 ml) in PBS were then added, the precipitate incubated for 20 min on ice, filtered through a glass-fibre filter (Whatman GF/C), washed with 10 ml of 15 % trichloroacetic acid and 10 ml of 95 % (v/v) ethanol, air-dried and counted in a scintillation counter. A control well was used in which [³H]thymidine was added just before isolation to assess background radioactivity.

Fluorescence-activated cell sorting (FACS) analysis

Rat VSM cells (5 × 10⁶) were cultured in DMEM containing 10 % (v/v) FCS for 48 h and each inhibitor added as above for 1, 2 or 3 days. Isolation of cells and processing for flow cytometry were as previously described (Bennett et al., 1994a).

Time-lapse cinemicroscopy

VSM cells were plated into 5-cm-diam. tissue-culture dishes (Nunc) at a density of 1 × 10⁴ cell/dish under sterile conditions. Cells were grown for 48 h in DMEM containing 10 % (v/v) FCS and then medium containing inhibitors was substituted. The dishes were then filmed using a time-lapse cinemicroscopy system as previously described (Bennett et al., 1994a) for 4 days.

Immunofluorescence analysis

VSM cells were plated on 8-well Tissue-Tek chamber slides (Nunc) and cultured for 48 h in DMEM containing 10 % FCS. The cells were then washed three times with PBS and the test medium added to each well 24, 16, 8, 4, 2, 1 and 0.5 h before isolation. Control cells (cultured in DMEM containing 10 % FCS) for each time point were treated similarly except that no inhibitor was added. All cells were processed for immunofluorescence analysis as previously described (Waters et al., 1991). Briefly, the cells were washed three times in PBS, fixed in 4% paraformaldehyde in PBS at room temperature for 15 min and then subjected to a sequential blocked incubation buffer (PBS, 1 % (v/v) BSA, 0.5 % Triton X-100, 0.02 % sodium azide) for 30 min at room temperature. C-Myc protein was detected with affinity-purified pan-Myc antibody (Moore et al., 1987), diluted 1/500 in incubation buffer, and bound antibody was visualized using fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG antibody (Amersham) at 1/200 dilution in incubation buffer. Specificity was ascertained by pre-absorption of pan-Myc antibody with peptide antigen and by incubation without the primary antibody. In neither case was fluorescence evident. Slides were viewed immediately after preparation on a Bio-Rad MRC500 confocal microscope using Nimbus image analysis software (Research Systems). Each experiment was repeated three times and image formation, collection and quantification performed using the same objective lens aperture, machine gain and blackness settings and with a Kalman filter averaging the image characteristics over 30 image scans. Cells (40 per treatment group) were analysed in two separate subconfluent fields of cells. Average intensity of fluorescence was calculated for each nucleus and the mean intensity of 40 nuclei used for data comparison. A similar process was used to assess cytoplasmic background staining for each cell and these figures were subtracted from the nuclear signal to yield a net C-Myc protein signal.

C-Myc protein quantification in defined medium and synchronized cells

VSM cells were plated on Tissue-Tek slides as above into defined medium (DM) [DMEM containing transferrin (5 μg/ml), selenite (30 nM), BSA (1 mg/ml) and fibronectin (1 mg/ml)]. This was supplemented with epidermal growth factor (EGF) (10 ng/ml), basic fibroblast growth factor (bFGF) (100 ng/ml), insulin (50 μg/ml) and platelet-derived growth factor AB (PDGF-AB) (10 ng/ml) (all supplied by Sigma). The cells were grown in this medium for 48 h and then the medium changed to one lacking PDGF, insulin, EGF or bFGF. Control cells were changed into medium containing all factors or DM alone. C-Myc protein immunofluorescence signals were determined for all cells after 2 h of treatment.

To synchronize VSM cells, double thymidine block or iso-leucine deprivation was used. Cells were cultured in DMEM with
10% (v/v) FCS and 2 mM thymidine for 16 h. The medium was then replaced with medium without thymidine for 9 h and then thymidine replaced for 16 h. The block was released by replacing with DMEM containing 10% (v/v) FCS for 4 and 10 h, which correspond to mid-S and mid-G2 phases in these cells respectively, as determined by flow cytometry (see above). The cells were then serum-starved (0.5% FCS) or treated with IFN-γ (100 i.u./ml) as above. For G1 synchronization cells were cultured in isoleucine-free DMEM containing 10% FCS for 48 h, and then serum-starved or treated with IFN-γ. The immunofluorescence signals were determined at each medium change and 2 h after serum reduction or IFN-γ treatment.

Measurement of c-Myc protein assay in bulk cultures

The use of the AMPAK e.i.s.a. system (Dako Diagnostics) for the analysis of c-Myc protein levels in cell lysates was a modified version (Waters et al., 1991) of that previously described (Moore et al., 1987). Protein concentrations were quantified against a bacterially expressed c-Myc protein standard p62c-myc.

RNA isolation, electrophoresis and Northern blotting

Subconfluent VSM cells grown in DMEM containing 10% (v/v) FCS were treated with growth inhibitors for 1, 2, 4, 8 or 24 h, washed three times in cold PBS, scraped into 50 ml of PBS and pelleted for 5 min at 1000 rev./min (200 g). Total RNA was isolated, electrophoresed, blotted and hybridized as previously described (Bennett et al., 1994b). c-myc mRNA was detected using a riboprobe derived from a linearized human c-myc cDNA coding for the deletion mutant D414-433 (Stone et al., 1987), and the signals normalized using a random-hexamer-primed rat glyceraldehyde phosphate dehydrogenase cDNA probe.

Statistical analyses

The means of cell number, [3H]thymidine incorporation and c-Myc protein levels were analysed using analysis of variance for multiple comparisons. Paired analysis between two groups, between control and an individual treatment group for instance, was performed using Student's t-test where ANOVA indicated significance for the multiple comparison.

RESULTS

Arrest of VSM cells following growth inhibitor treatment

We first investigated the effect of a range of pharmacological inhibitors of VSM proliferation and serum reduction on the arrest of exponentially proliferating VSM cells. Serum reduction and all of the pharmacological inhibitors tested markedly reduced cellular proliferation, as assessed by live cell number, [3H]thymidine incorporation (Figure 1), BrdU (bromodeoxyuridine) incorporation and FACS analysis (Figure 2 and Table 1) and time-lapse cinemicroscopy (results not shown). Statistically significant growth arrest occurred by day 6 in all treatment groups. Exponentially growing cells showed a relatively constant level of [3H]thymidine incorporation per cell during the course of the experiment, showing that most cells were in the cell cycle over this period. Pulse BrdU labelling and DNA content analysis (Figure 2 and Table 1) showed progressive loss of S-phase cells between 1 and 3 days in all treatment groups and concomitant accumulation in G1/G0. Additionally, in both the heparin- and IFN-γ-treated cells there was some accumulation in G2 (Table 1). There was no evidence of cell death with any inhibitor treatment, as assessed by time-lapse cinemicroscopy (results not shown).

C-myc expression following serum reduction

Following treatment with the growth inhibitors, c-Myc protein expression was determined at the single-cell level by semi-quantitative immunofluorescence (Figures 3–6) and confirmed in bulk populations by e.i.s.a. (Table 2). Both were in good agreement (correlation coefficient 0.78), indicating that changes in c-Myc protein expression do not exhibit substantial heterogeneity among cells. In log-phase cells in 10% (v/v) FCS, c-Myc protein is evident by its characteristic nuclear staining with nucleolar sparing (Figure 3). Staining is lost completely upon pre-absorption of the antibody with the appropriate peptide immunogen (results not shown), indicating antibody specificity.
Serum reduction led to a rapid fall in c-Myc protein signal within 2 h in all cells, and this persisted while low-serum conditions were maintained (Figures 3 and 6). A similarly rapid fall in c-myc mRNA was seen following serum reduction (Figure 7).

**C-myc expression in defined medium (DM)**

In order to determine if any specific growth factor in serum is absolutely required to maintain c-Myc protein expression, VSM cells were cultured in DM containing PDGF, bFGF, EGF and insulin and then transferred to defined media lacking an individual growth factor. Reduction in c-Myc protein signal was observed after transfer to medium lacking PDGF, bFGF and EGF, but no effect was seen after transfer to medium lacking insulin (Table 3). Thus no single growth factor is required to maintain c-myc expression and PDGF, EGF and bFGF all contribute to some extent. Transfer to defined medium alone without PDGF, bFGF, EGF and insulin rapidly reduced c-Myc protein signal to approx. 20% of the level seen in control cells (Table 3). Transfer of cells from medium containing 10% (v/v)
C-myc and vascular smooth-muscle cell inhibition

Treatment with 0.5% FCS

Time 0 | 30 min

60 min | 120 min

Figure 3  Indirect immunofluorescence of VSM cells stained for c-Myc protein, showing the characteristic nuclear pattern with nucleolar sparing

The time course of immunofluorescence signal shows loss of signal over a 2 h period of treatment with 0.5% FCS. The scale bar represents 5 μm.

Figure 4  Indirect immunofluorescence time course of c-Myc protein signal showing loss of signal over a 4 h period following treatment with IFN-γ (100 i.u./ml)

Down-regulation of c-Myc signal occurs in each cell. The scale bar represents 25 μm.

Figure 5  Indirect immunofluorescence time course of c-Myc protein signal following treatment with heparin (50 μg/ml), 8-Br-cAMP (0.1 mM) and 8-Br-cGMP (0.1 mM)

No change in signal was observed after 2 h and positive cells were still present at 24 h of treatment. The scale bar represents 5 μm.

Figure 6  Graph of c-Myc protein immunofluorescence signal against time after treatment for each inhibitor

Results were expressed as percentages of the signal derived from control cells (cultured in DMEM containing 10% FCS) at the same time points (n = 3).

Table 2  C-Myc protein concentration in molecules/cell for VSM cells following inhibitor treatment, measured by e.i.l.s.a.

Cells were cultured in DMEM containing 10% FCS for 48 h (Control cells), and then medium was changed for one containing inhibitors as shown. Values given are means (S.E.M.), n = 3.

*P < 0.05, **P < 0.01, versus Control cells at the same time point. Correlation coefficient of the immunocytochemical assay versus e.i.l.s.a. = 0.78.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>C-Myc protein concn. (molecules/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Control cells</td>
<td>3700 (475)</td>
</tr>
<tr>
<td>0.5% FCS</td>
<td>300 (50)**</td>
</tr>
<tr>
<td>Heparin (50 μg/ml)</td>
<td>3550 (375)</td>
</tr>
<tr>
<td>8-Br-cAMP (0.1 mM)</td>
<td>3800 (250)</td>
</tr>
<tr>
<td>8-Br-cGMP (0.1 mM)</td>
<td>3650 (450)</td>
</tr>
<tr>
<td>IFN-γ (100 i.u./ml)</td>
<td>1150 (250)*</td>
</tr>
</tbody>
</table>
Table 3  C-Myc protein fluorescence signals from VSM cells cultured in medium lacking a single defined growth factor

Results were expressed as a percentage of the signal from control cells (cultured in DM + all growth factors). Cells were cultured in DM containing PDGF, bFGF, EGF and insulin for 48 h, and then transferred to medium without one growth factor, or DM alone. Fluorescence signals were assessed 2 h after transfer. The average nuclear signal for each treatment group was calculated from 40 cells/group. Values given are means (S.E.M.), n = 3. Statistical significance:

*P < 0.05, **P < 0.01 versus control cells.

<table>
<thead>
<tr>
<th>Culture medium</th>
<th>Relative c-Myc protein level (% of control signal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defined medium (DM) + all factors</td>
<td>100</td>
</tr>
<tr>
<td>PDGF omitted</td>
<td>62.2 (4.5)*</td>
</tr>
<tr>
<td>EGF omitted</td>
<td>53.9 (4.9)*</td>
</tr>
<tr>
<td>bFGF omitted</td>
<td>61.1 (6.3)*</td>
</tr>
<tr>
<td>Insulin omitted</td>
<td>96.1 (6.8)</td>
</tr>
<tr>
<td>DM alone</td>
<td>19.3 (3.2)**</td>
</tr>
<tr>
<td>Medium containing 10% FCS</td>
<td>120.0 (4.8)*</td>
</tr>
</tbody>
</table>

FCS to one containing all growth factors showed that PDGF, bFGF and EGF accounted for approx. 80% of the signal seen in 10% FCS.

C-myc expression after IFN-γ treatment

Addition of IFN-γ to proliferating VSM cells caused rapid loss of c-Myc protein signal, although to a lesser degree than serum reduction. This occurred in every cell and was maintained in the continued presence of IFN-γ (Figures 4 and 6). C-myc mRNA showed a similar rapid reduction after IFN-γ treatment (Figure 7).

C-myc expression in synchronized cells

To investigate the cell-cycle-dependence of c-myc down-regulation, synchronization of the cells in G1, S and G2 phases was performed by isoleucine deprivation and release from double thymidine block. This showed that 2 h after serum reduction and IFN-γ treatment c-Myc protein fluorescence fell by a similar amount in VSM cells in each phase of the cell cycle (Table 4). Thymidine block and isoleucine deprivation themselves also reduced c-Myc protein expression, although expression returned to the levels seen in control cells 2 h after release from thymidine block.

C-myc expression after heparin and cyclic nucleotide analogue treatments

In contrast with serum reduction and IFN-γ treatments, heparin, 8-Br-cAMP and 8-Br-cGMP treatments caused no significant change in c-Myc protein immunofluorescence within the first 2 h, although a loss of signal is evident by 24 h (Figures 5 and 6), which was confirmed on c-Myc protein e.l.i.s.a. (Table 2). Analysis of c-myc mRNA by Northern blot analysis in each case yielded a similar pattern to that seen with c-Myc protein, and further decreases in c-myc mRNA were evident by 48 h (Figure 7). In view of the delayed down-regulation of c-myc following treatment with these agents, synchronization studies were not performed.

Figure 7  Northern-blot hybridization of c-myc mRNA following treatment with each inhibitor

(a) 0–24 h; (b) 0–48 h. Equal loading of RNA/lane was confirmed by the approximately equal signals from the glyceraldehyde-phosphate dehydrogenase (GAPDH) exposures. The IFN-γ signals at 0–48 h in (b) were obtained by exposure of the filters for approximately four times as long as with the other inhibitors shown over this time period or in (a), confirming that changes in c-myc mRNA expression after IFN-γ treatment occur early, over the first 0–4 h.
Table 4  C-Myc protein fluorescence signals from VSM cells synchronized by thymidine block or isoleucine deprivation, expressed as a percentage of the signal obtained from control cells (cultured in DMEM containing 10% FCS alone)

Cells were synchronized by double thymidine block (2 mM thymidine), and release for 4 h (mid S-phase) or 10 h (mid G2), or by isoleucine deprivation (G1). Release from thymidine block was performed by culturing in medium without thymidine. This was followed by serum reduction or IFN-γ treatment, and signals assessed after 2 h. Statistical significance: *P < 0.05; **P < 0.01 versus control cells.

<table>
<thead>
<tr>
<th>Synchronization treatment</th>
<th>Relative c-Myc protein level (% of control signal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymidine block (TB)</td>
<td></td>
</tr>
<tr>
<td>Control cells</td>
<td>100</td>
</tr>
<tr>
<td>TB +4 h release</td>
<td>69.2 (5.3)*</td>
</tr>
<tr>
<td>TB +4 h release + 2 h 0.5% FCS</td>
<td>98.4 (6.7)</td>
</tr>
<tr>
<td>TB +4 h release + 2 h IFN-γ</td>
<td>18.8 (2.6)**</td>
</tr>
<tr>
<td>TB +10 h release</td>
<td>95.6 (11.2)</td>
</tr>
<tr>
<td>TB +10 h release + 2 h 0.5% FCS</td>
<td>18.3 (3.4)**</td>
</tr>
<tr>
<td>TB +10 h release + 2 h IFN-γ</td>
<td>25.8 (6.3)**</td>
</tr>
<tr>
<td>Isoleucine deprivation (ISLD)</td>
<td></td>
</tr>
<tr>
<td>Control cells</td>
<td>100</td>
</tr>
<tr>
<td>ISLD +2 h 0.5% FCS</td>
<td>64.5 (11.1)*</td>
</tr>
<tr>
<td>ISLD +2 h IFN-γ</td>
<td>11.5 (3.7)**</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The regulation of VSM proliferation in the normal arterial wall and in atherosclerotic plaques is considered central to the pathogenesis of atherosclerosis (Ross, 1993). Limited availability of mitogens, the action of endogenously induced anti-proliferative cytokines like IFN-γ, growth-inhibitory interactions with heparin-like glycosaminoglycans, and agents that elevate intracellular cyclic nucleotide levels probably all contribute to restricting VSM cell proliferation in vivo. Each of these processes can be mimicked in vitro, for example by serum deprivation, by addition of IFN-γ, of heparin, or of cyclic nucleotide analogues respectively. In many cell types, growth arrest in response to mitogen withdrawal (Dean et al., 1986; Waters et al., 1991), growth-inhibitory cytokines such as IFN (Jonak and Knight, 1984; Einat et al., 1985a, b) or TGF-β (Pietenpol et al., 1990) is accompanied by rapid down-regulation of c-myc expression. This suggests that c-myc expression is tightly linked to continued cell proliferation. VSM cells, like fibroblasts, continuously express c-myc during proliferation, but little is known of the effects of growth inhibitors on c-myc expression in exponentially proliferating cells. We therefore investigated whether c-myc down-regulation occurs in proliferating VSM cells following treatment with a variety of growth inhibitors thought to differ in their modes of action. A role for down-regulation of c-myc in VSM proliferation would be particularly interesting in view of the evidence that c-myc is overexpressed in VSM cells derived from atherosclerotic plaques (Parkes et al., 1991).

We find that serum reduction, IFN-γ, heparin and cyclic nucleotide analogues all arrest exponentially proliferating VSM cells in the G1/G0 phase of the cell cycle. In the presence of heparin and IFN-γ some accumulation of cells is also evident in G2. Both serum deprivation and IFN-γ led to the rapid down-regulation of c-Myc protein and mRNA. C-Myc protein down-regulation occurs in every cell and, as the cell populations studied were asynchronous, appears independent of cell-cycle position.

This was confirmed by examining c-myc down-regulation in cell populations synchronized in G1, S and G2 phases; c-Myc protein levels fell to similar levels in each phase. We conclude that expression of c-myc in proliferating VSM cells is dependent on the continuous presence of mitogens, but may be reduced by the presence of IFN-γ at any point in the cell cycle. Interestingly cells do not arrest at the point at which c-myc was down-regulated, rather cells arrest in G1 following serum reduction and G2 and G0 following IFN-γ treatment. Previously it has been proposed that cells require mitogens up to a restriction point within the cell cycle, beyond which cells can complete the cycle without mitogenic stimuli (Pardee, 1989). In contrast, we have found that cells are capable of responding to growth arrest signals throughout the cell cycle. The rapid disappearance of c-Myc protein may therefore represent a major signalling pathway for the arrest process following serum removal or IFN-γ exposure, but the actual site of arrest may be determined by other factors, such as the treatment used.

The role of individual growth factors to act independently to maintain steady-state c-myc expression was examined using defined medium supplemented with growth factors. Un-synchronized cells transferred to medium lacking PDGF, bFGF and EGFR show a reduction in c-Myc protein fluorescence in each cell; the maintained expression of c-myc in proliferating VSM cells in 10% FCS thus appears dependent on the presence of a combination of mitogens, and PDGF, bFGF and EGF account for approx. 80% of c-Myc protein expression.

In contrast with serum reduction and IFN-γ, heparin and cyclic nucleotide analogues do not down-regulate c-myc rapidly, despite exhibiting similar overall inhibition of proliferation at 3–9 days. Instead, gradual down-regulation of c-myc expression becomes evident after some 8–24 h. However, that this suppression of c-Myc is important for growth arrest after heparin and cyclic nucleotides treatment has been shown by the fact that VSM cells which have deregulated c-myc expression at the level seen in log-phase cells do not arrest following these treatments (Bennett et al., 1994a). Growth arrest of VSM cells therefore does not appear to require complete suppression of c-Myc; rather suppression below the level seen in log-phase cells is sufficient. We conclude that distinct signalling pathways involving c-myc exist for inhibition of VSM cell proliferation by mitogen deprivation and IFN-γ, on the one hand, and by heparin and cyclic nucleotide analogues on the other. Both c-Myc protein and mRNA possess very short half-lives, typically 20–30 min. The down-regulation of c-Myc protein and mRNA which we observe in VSM cells in response to both mitogen deprivation and IFN-γ is therefore consistent with imposition of an immediate block to new c-myc expression and the decay of pre-existing c-Myc protein and mRNA. Both mitogen withdrawal and IFN-γ thus seem to exert a direct effect on c-myc expression and the delayed reduction in c-Myc protein and mRNA following treatment with heparin, 8-Br-cAMP or 8-Br-cGMP may thus indicate an indirect effect. The mechanism by which any of these inhibitors reduce c-myc expression is unclear although interference treatment and serum reduction have been found to directly reduce c-myc transcription and decrease mRNA stability in other cells (Dani et al., 1985; Einat et al., 1985b; Dean et al., 1986).

In conclusion, we have described two discrete mechanisms of c-myc down-regulation associated with inhibition of proliferation in VSM cells, one of which involves a profound early decrease in c-Myc protein levels. However, c-myc down-regulation accompanies growth arrest from all agents used here, including mitogen withdrawal, a number of pharmacological agents and metabolic block. This implies that continuous low-level expression of c-myc is tightly linked to proliferation in VSM cells and that down-
regulation of c-Myc may act as a signal for growth arrest. This is in agreement with our previous data which showed that deregulated expression of c-myc promotes inappropriate VSM cell proliferation (Bennett et al., 1994a) and which suggested that c-Myc down-regulation is a pre-requisite for growth arrest in VSM cells. Overexpression of c-myc, as has been described in human atheromatous plaque VSM cells (Parkes et al., 1991), may thus be an important factor in the VSM cell accumulation seen in atherosclerosis.

This study was supported by a British Heart Foundation Clinical Scientist Research Fellowship (M.R.B.). We would like to thank Mr. Derek Davies for technical assistance with the flow cytometry.

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Received 18 January 1994/8 March 1994; accepted 22 March 1994