Hexamethylenebisacetamide selectively inhibits the proliferation of human and rat vascular smooth-muscle cells

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Hexamethylenebisacetamide (HMBA) selectively and reversibly inhibited proliferation of human and rat vascular smooth-muscle cells (VSMCs) compared with endothelial cells, fibroblasts or lymphocytes. Half-maximal inhibition of VSMC proliferation occurred at 2–5 mM-HMBA, and at 30–> 50 mM for other cell types. HMBA also prevented de-differentiation, defined by the loss of smooth-muscle-specific myosin heavy chain, of primary rat VSMCs and caused partial re-differentiation of subcultured cells. Other inhibitors of ADP-ribosyltransferase were also selective inhibitors of VSMC proliferation.

INTRODUCTION

Abnormal proliferation of VSMCs is a major component of vascular disease, including atherosclerosis, vascular rejection and re-stenosis following angioplasty [1,2]. Intimal VSMC proliferation in vascular disease is thought to result from endothelial dysfunction or injury [3,4]. Agents which selectively inhibit VSMC proliferation without affecting endothelial repair might therefore be expected to prevent or inhibit the progress of these diseases.

When arterial VSMCs are dispersed into cell culture, they begin to lose smooth-muscle-specific isoforms of MHC and actin and accumulate their non-muscle equivalents before proliferating [5,8]. VSMCs in the de-differentiated state *in vitro* resemble the cells found in atheromatous plaques [3], and it has been proposed that de-differentiation is an essential precursor to proliferation [9]. If this hypothesis is correct, the de-differentiation process offers a potential target for the selective inhibition of VSMC proliferation, since a variety of other types of cells proliferate without apparently de-differentiating.

HMBA is the best-characterized compound known to inhibit ADPRT [NAD⁺:poly(ADP-D-ribose) ADP-D-ribosyltransferase, EC 2.4.2.30] [10,11]. It has been shown to cause morphological and functional differentiation of several transformed cell lines and to inhibit their proliferation *in vitro* [12–14] and *in vivo* [15]. These observations provided the rationale for examining the effects of HMBA on the morphology and de-differentiation of VSMCs defined by the loss of smooth-muscle-specific MHC and for comparing its effects on the proliferation of VSMCs and other types of cells. This comparison was focused on cells involved in endothelial repair, wound healing and the immune response, which must remain able to proliferate if an inhibitor of VSMC proliferation is to be of practical use. The mechanism of inhibition of VSMC proliferation by HMBA was explored by comparing its selectivity as an inhibitor of cell proliferation with that of other inhibitors of ADPRT. We have also examined when HMBA must be present in the cell cycle to inhibit the proliferation of VSMCs.

MATERIALS AND METHODS

Cell cultures and assay of DNA synthesis

Rat VSMCs were cultured after enzymic dispersion of aortic media as described previously [5]. The cells were maintained in medium M199 (ICN/Flow) with 10% (v/v) FCS (ICN/Flow). Human VSMCs were cultured after enzymic dispersion of aortic media (as for rat tissue) obtained from transplant donors. The human cells were cultured in Dulbecco's modified Eagle's medium (DMEM; ICN/Flow) with 20% FCS. Endothelial cells from human umbilical vein were kindly given by Amanda Wrenn (Department of Pharmacology, Cambridge, U.K.) and were maintained in medium M199 with 15% FCS on plates coated with 0.2% gelatin (Sigma). Rat aortic endothelial cells were isolated from the thoracic portion of rat aortae by digestion in collagenase (1 mg/ml) from Sigma in serum-free M199 medium for 7 min at 37 °C. The cells were collected by centrifugation (2700 g; 3 min), and the pellet was resuspended in M199 containing 15% FCS and plated out at a cell density equivalent to the yield from one aorta per 2 cm² in dishes treated with 0.2% gelatin for 45 min. Non-adherent blood cells were washed out after 24 h, when the endothelial cell density was 3 × 10⁴ cells/cm². Rat and human fibroblasts were cultured after enzymic dispersion of aortic adventitia as for VSMC culture and maintained in M199 with 10% FCS. Rat hepatocytes were dispersed by collagenase infusion as described previously [16] and maintained in DMEM with 20% FCS. Rat thymus- and spleen-derived lymphocytes were cultured as described previously [17] and maintained in M199 with 10% FCS plus 5 µg of concanavalin A/ml (Sigma) as a mitogen. All experiments on primary cells are timed from plating out (0 h).

Subcultured cells were passaged (passage number in parentheses) by releasing the cells from the dish with trypsin/EDTA solution (Gibco) and re-plating the cells at a 1:2 dilution in medium. Subcultured cells were made quiescent in serum-free M199 for 48 h before re-stimulation by addition of FCS (timed as 0 h for all subcultured-cell experiments). All types of primary and subcultured cells were incubated in 5% CO₂ in air at 37 °C.

Abbreviations used: ADPRT, ADP-ribosyltransferase (EC 2.4.2.30); FCS, foetal-calf serum; HMBA, hexamethylenebisacetamide; MHC, myosin heavy chain; SM-1 and SM-2, 204 kDa and 200 kDa smooth-muscle-specific MHC isoforms respectively; VSMC, vascular smooth-muscle cell; ID₅₀, concn. giving 50% inhibition.

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Fig. 1. Effect of HMBA on DNA synthesis

(a) Effect of HMBA on $[^3\text{H}]$ thymidine incorporation in human VSMCs and endothelial cells: $\square$, primary VSMCs in 20% FCS ($[^3\text{H}]$ thymidine incorporation from 24 to 72 h; control cells 23 625 c.p.m.); $\bullet$, subcultured human endothelial cells in 15% FCS (12–36 h; control cells 71 026 c.p.m.). (b) Effect of HMBA on $[^3\text{H}]$ thymidine incorporation in rat VSMCs and endothelial cells: $\square$, primary VSMCs in 10% FCS (24–60 h; control cells 28 490 c.p.m.); $\bullet$, subcultured rat endothelial cells in 15% FCS (12–36 h; control cells 30 315 c.p.m.). (c) Effect of removal of 10 mM-HMBA at 72 h on $[^3\text{H}]$ thymidine incorporation in rat primary VSMCs: $\bullet$, cells in the presence of 10 mM-HMBA throughout; $\blacksquare$, cells in the presence of 10 mM-HMBA until 72 h; $\square$, control cells in the absence of HMBA. (d) Effect of addition of 10 mM-HMBA at 72 h on $[^3\text{H}]$ thymidine incorporation in rat primary VSMCs: $\bullet$, cells in the presence of 10 mM-HMBA throughout; $\blacksquare$, cells in the presence of 10 mM-HMBA from 72 h onwards; $\square$, control cells in the absence of HMBA.

Table 1. HMBA concentrations causing half-maximal inhibition of DNA synthesis (ID_{50})

HMBA was added to primary cell cultures on plating out. Cells were subcultured (passage number in parentheses) and HMBA was added on serum deprivation for 48 h before re-stimulation with serum as described in the Materials and methods section. ID_{50} values were determined from dose-response data as shown in Fig. 1. The range of values for each cell type was obtained from 3–5 experiments, except for primary and early passage rat VSMCs ($n > 9$) and primary rat liver cells ($n = 2$).

Species	Cell type	ID_{50} (mM)
Human	Primary VSMC	3–5
	Subcultured (3) VSMC	2–5
	Subcultured (4) adventitial fibroblasts	30–50
	Subcultured (6) endothelial cells	30–40
Rat	Primary VSMC	3–5
	Subcultured (4) VSMC	2–4
	Subcultured (26) VSMC	1–2
	Primary adventitial fibroblasts	40–50
	Subcultured (4) adventitial fibroblasts	30–50
	Subcultured (3) endothelial cells	> 50
	Primary thymus derived lymphocytes	30–50
	Primary spleen derived lymphocytes	30–50
	Primary liver cells	30–40

HMBA, coumarin (1,2-benzopyrone) or 4-hydroxyquinazoline (all from Sigma Chemical Co.) were added from stock solutions in sterile water, of 1 M, 50 mM and 5 mM respectively, to primary

cell cultures on plating out at 0 h. The inhibitors were added to subcultured cells at the time they were incubated in FCS-free M199 to render them quiescent.

DNA synthesis was assayed by $[^3\text{H}]$ thymidine incorporation for the periods indicated as described previously [5]; values are means of triplicate determinations $\pm$ S.E.M. Preliminary experiments were performed to determine for each cell type when the cells first entered S phase after addition of serum and when the first cell cycle was complete by cell counting. The time of incubation with $[^3\text{H}]$ thymidine for each cell type was adjusted to include the entire transition from the initiation of first S phase to the end of first M phase. This ensured that the sensitivity of the various cell types to inhibition of DNA synthesis by HMBA and the other inhibitors was compared for completed passages through the first cell cycle.

Analysis of MHC isoforms

Western-blot analysis of smooth-muscle-specific MHC isoforms was performed as described previously [5]; briefly, samples of 4×10^6 cells were lysed, separated by lithium dodecyl sulphate/PAGE (3–5% acrylamide gels), blotted on to nitrocellulose and made visible by direct immunoperoxidase staining using an antiserum to chicken gizzard smooth-muscle myosin which recognizes SM-1 and SM-2 (obtained from Professor Ute Groschel-Stewart, Heidelberg, Germany; see [18]). The Western blots were quantified by using a u.v. scanning densitometer. Direct immunoperoxidase staining of smooth-muscle-specific MHC in cells was performed by fixing the cells in 70% (v/v) ethanol containing 1% acetic acid for 30 min at 4°C, and incubating with the antiserum at 10 $\mu\text{g}/\text{ml}$ for 2 h, followed by peroxidase-linked second antibody for 1 h.

Table 2. ID₅₀ values for 4-hydroxyquinazoline and coumarin

ID₅₀ values were determined as described for HMBA in Table 1. The range of values for each type of cell was obtained from 3 experiments, except for primary liver cells ($n = 2$).

Inhibitor	Species	Cell type	ID ₅₀ (μ M)
4-Hydroxyquinazoline	Rat	Primary VSMC	15–25
	Rat	Subcultured (4) adventitial fibroblasts	300–400
	Rat	Primary thymus-derived lymphocytes	200–300
	Rat	Primary liver cells	300–500
	Human	Subcultured (6) endothelial cells	200–300
Coumarin	Rat	Primary VSMC	60–80
	Rat	Subcultured (4) adventitial fibroblasts	800–1000
	Rat	Primary thymus-derived lymphocytes	~ 2000
	Rat	Primary liver cells	800–1000
	Human	Subcultured (6) endothelial cells	800–1000

RESULTS AND DISCUSSION

The concentration of HMBA required to inhibit DNA synthesis in freshly dispersed primary VSMCs in culture ($t = 0$ h) was lower than for endothelial cells by a factor of 8 for human cells (Fig. 1a) and by a factor of > 15 for rat cells (Fig. 1b). DNA synthesis in subcultured human or rat VSMCs was inhibited by 50% at a similar concentration (ID₅₀) to that for the corresponding primary cells (Table 1). Human or rat fibroblasts, lymphocytes and rat liver cells all had ID₅₀ values similar to those for endothelial cells (Table 1). The inhibition of DNA synthesis in primary rat VSMCs by 10 mM-HMBA was maintained for at least 144 h, when the increase in cell number was decreased by $85.4\% \pm 5.1\%$. The effect of HMBA was fully reversible, and [³H]thymidine incorporation reached the level of incorporation in untreated cells within 48 h after the removal of 10 mM-HMBA (Fig. 1c). These data show that inhibition of VSMC proliferation can be maintained by HMBA for as long as it is present in the medium, and that inhibition is not due to cytotoxic effects.

The experiments described showed that HMBA would prevent the entry into the cell cycle of freshly isolated non-proliferating VSMCs. HMBA was also added to exponentially growing rat VSMCs in primary culture at 72 h (Fig. 1d) and caused maximal inhibition of [³H]thymidine incorporation within one cell cycle (35 h [5]). Thus HMBA causes exit of proliferating cells from the cell cycle.

Further experiments were performed to determine whether HMBA would inhibit proliferation in response to stimulation by defined growth factors. HMBA (10 mM) inhibited DNA synthesis in primary rat VSMCs to the same extent whether the cells were stimulated with FCS, epidermal growth factor, basic fibroblast growth factor or the BB dimer of platelet-derived growth factor. The inhibition of proliferation by HMBA is therefore independent of the mitogenic agent by which the cells are activated to enter the cell cycle.

More potent inhibitors of ADPRT than HMBA have been described, including coumarin [10,15] and 4-hydroxyquinazoline [19], and they were therefore examined for selective effects on VSMC proliferation. These agents had 12–15-fold selectivity for rat VSMCs compared with endothelial cells as inhibitors of proliferation, similar to the selectivity obtained with HMBA (Table 2). These data are consistent with the working hypothesis that inhibition of ADPRT is the mechanism by which these agents selectively inhibit VSMC proliferation.

The effects of HMBA on the de-differentiation of VSMCs were

also examined. In previous studies it has been shown that in freshly dispersed rat VSMCs smooth-muscle-specific MHC is present as equal amounts of the 204 kDa (SM-1) and 200 kDa (SM-2) isoforms [5,7] (e.g. see 0 h lane in Fig. 2a). Under the culture conditions used, primary rat VSMCs lost both of the MHC isoforms in either the presence (Fig. 2b) or the absence of FCS [6], i.e. independently of mitogenic stimulation. On reaching stationary phase after 144–168 h in the presence of FCS [5], proliferation ceased and SM-1 accumulated to approx. 20% of the amount in freshly dispersed cells and to approx. 40% after the withdrawal of FCS (lanes 2 and 3 in Fig. 2d). However, there was little or no re-expression of SM-2 in the cells at stationary phase, and the ratio of SM-1 to SM-2 was approx. 8:1, compared with approx. 1:1 in the freshly dispersed cells (Fig. 2d).

Primary rat VSMCs treated with 10 mM-HMBA in the presence or absence of serum maintained very similar amounts of SM-1 and SM-2 at 144 h to those present in the freshly dispersed cells (Figs. 2a and 2b). Direct immunoperoxidase staining of smooth-muscle-specific MHC in primary cell cultures after 144 h in the presence and absence of 10 mM-HMBA also showed the retention of the protein in cells treated with HMBA (Fig. 2e). However, 10 mM-HMBA had little effect on the re-accumulation of smooth-muscle-specific MHCs when added at stationary phase to primary cultures either in the presence of FCS or after its withdrawal (lanes 2 and 4 and lanes 3 and 5 in Fig. 2d).

Early-passage rat VSMCs, which contain relatively small amounts of smooth-muscle-specific MHCs (0 h lane in Fig. 2c) and have lost the ability to re-accumulate significant amounts of the proteins at stationary phase, re-accumulated approx. 30% of the amount of the proteins present in freshly dispersed cells when 10 mM-HMBA was added to the exponentially growing cells for 24 h (Fig. 2c). Under these conditions, SM-1 and SM-2 were re-expressed in approximately equal proportions, as in the freshly dispersed cells. In addition to the SM-1 and SM-2 isoforms, there was a third band which reacted with antisera to smooth-muscle-specific myosin (labelled X) and which had the same molecular mass as the recently described SM-3 isoform [20] (Fig. 2c).

To determine when in the cell cycle HMBA exerted its inhibitory effect on proliferation, subcultured VSMCs were made quiescent by serum deprivation for 48 h before re-stimulation with serum, and DNA synthesis was assayed after 20–24 h. Full inhibition of DNA synthesis only occurred when HMBA was present within the first 6 h after the re-addition of serum, and there was no significant effect on DNA synthesis when HMBA was added 12 h after serum (Fig. 3a). This implies that HMBA inhibits early events in the G₁ phase of the cell cycle, but is not

Fig. 2. Effect of HMBA on the differentiation of rat VSMCs

(a) SM-1 and SM-2 in primary rat VSMCs cultured in M199 with 10% FCS in the presence of 10 mM-HMBA from 0 h to 144 h, assayed by Western blotting with antiserum to smooth-muscle myosin as described in the Materials and methods section. (b) Total smooth-muscle-specific MHC (SM-1 + SM-2; SM-MHC) content per cell relative to the amount at 0 h, determined from the densitometric traces of the lanes in (a) (■). Loss of SM-1 + SM-2 in control cell cultures without HMBA (□). (c) Time course over 24 h of SM-1 and SM-2 accumulation in subcultured (passage 4) rat VSMCs in M199 with 10% FCS determined by Western blotting. HMBA (10 mM) was added at 0 h to cells in exponential growth. A third protein, in addition to SM-1 and SM-2, cross-reacting with the antiserum to smooth-muscle-specific myosin is labelled X (see the text). An equivalent Western blot from freshly dispersed primary cells (P) is shown for comparison. (d) SM-1 and SM-2 in primary rat VSMCs assayed by Western blotting. Lane 1, freshly dispersed primary cells (P) at 0 h. Lanes 2–5, stationary-phase primary cells harvested at 196 h after dispersion. Lane 2, cells in M199 with 10% FCS; lane 3, cells in FCS-free M199 from 144 h; lane 4, cells in M199 with 10% FCS with 10 mM-HMBA added at 144 h; lane 5, cells in FCS-free M199 with 10 mM-HMBA from 144 h. (e) Direct immunoperoxidase staining of primary rat VSMCs stained with antiserum to smooth-muscle myosin after 144 h in the presence or absence of HMBA. The cells treated with HMBA are more sparse because they have not proliferated significantly (magnification $\times 900$).

effective later in G_1 before DNA synthesis can first be detected at approx. 15 h after re-stimulation by serum. HMBA also affected the morphology of the cells, causing them to assume a more extended spindle form if added within 6 h of re-stimulation with serum, but had little effect when added later (Fig. 3b).

Taken together, these data show that inhibition of rat VSMC proliferation by HMBA is correlated with retention of SM-1 and SM-2 in primary cells and with re-expression of these proteins in passaged cells of altered morphology. This correlation between smooth-muscle-specific MHC and proliferation is consistent with

the view that de-differentiation is an essential precursor to proliferation [9]. However, other work has shown that heparin causes retention of smooth-muscle-specific MHCs in primary rat VSMCs, but does not prevent the eventual proliferation of the cells, although the length of the cell cycle is increased (D. J. Grainger, C. M. Wittchell, J. C Metcalfe & P. L. Weissberg, unpublished work). Further evidence that de-differentiation as defined by the loss of smooth-muscle-specific MHC is not obligatory before proliferation has been obtained from clones derived from single primary rat VSMCs, which retain smooth-

Fig. 3. Effect of delayed addition of HMBA on DNA synthesis and morphology of re-stimulated rat VSMCs

(a) Subcultured rat VSMCs were made quiescent by serum deprivation for 48 h before re-stimulation by addition of 10% FCS as described in the Materials and methods section. HMBA (10 mM) was added to replicate cell cultures at intervals after addition of FCS, and DNA synthesis was assayed by [3 H]thymidine incorporation from 20 to 24 h. DNA synthesis is expressed relative to control cells to which no HMBA was added. (b) Effect on cell morphology of addition of 10 mM-HMBA at intervals after addition of 10% FCS (magnification $\times 450$). Photographs were taken 24 h after stimulation.

muscle MHC throughout many rounds of division (D. J. Grainger, unpublished work). These observations and the data for HMBA are consistent with the alternative hypothesis that we have proposed [5], that de-differentiation, as defined by the smooth-muscle-specific MHC content of the cells, normally occurs in parallel with, but is not essential for, proliferation. We conclude that ADPRT inhibitors are likely to be powerful tools with which to analyse the relationship between differentiation and proliferation in VSMCs, and may be prototypes for the development of clinical agents to control VSMC proliferation.

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