Control of vascular smooth-muscle cell growth by macrophage-colony-stimulating factor

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Since in several vascular diseases abnormal vascular smooth-muscle cell (VSMC) proliferation is often associated with the presence of macrophages, we examined whether macrophage-colony-stimulating factor (M-CSF) might play a role in the control of VSMC growth. VSMCs were isolated from rat aorta and maintained in culture. Using a bioassay, a macrophage-colony-stimulating activity was detected in the serum-free supernatant of VSMCs, which could be inhibited by the addition of specific anti-M-CSF antibodies. The presence of M-CSF receptor protein and of M-CSF and M-CSF receptor gene transcripts was demonstrated by immunocytochemistry, using a specific anti-c-Fms antibody and Northern blot analysis respectively. [³H]Thymidine incorporation was measured following the addition to quiescent VSMCs of various dilutions of L929 cell supernatant (as a source of M-CSF) or of recombinant M-CSF.

Both exogenous M-CSF and serum-free VSMC conditioned medium promoted DNA synthesis in a concentration-dependent manner, and this effect could be abrogated by the presence of a specific anti-M-CSF antibody. Under similar experimental conditions, L929 cell supernatant modulated proto-oncogene expression, as assessed by Northern blot analysis of *c-fos*, *c-myc*, *egr-1* and *junB*. It was further demonstrated that M-CSF could act in synergy with thrombin, platelet-derived growth factor or basic fibroblast growth factor in promoting VSMC DNA synthesis. These results support the hypothesis that M-CSF affects the growth of cultured rat VSMCs through paracrine/autocrine mechanisms. Its effects at both the macrophage and the VSMC level confer to M-CSF a central role in the development of vascular lesions that occurs during atherosclerotic progression.

INTRODUCTION

Abnormal accumulation of vascular smooth-muscle cells (VSMCs) is a prominent feature of several vascular diseases, including atherosclerosis and restenosis following balloon angioplasty (see [1] for a review). This reflects excessive cell proliferation in response to growth factors and/or a failure to arrest in response to growth inhibitors [2]. There is, therefore, considerable interest in elucidating the mechanisms involved in excessive VSMC proliferation [3,4].

Atherosclerotic lesions, in which VSMCs account for the majority of cells, also contain monocyte/macrophage and T lymphocyte infiltrates which produce a number of proteins, including cytokines and growth factors, that are able to alter the physiological responses of the vascular wall [1]. Among these factors, the macrophage-colony-stimulating factor (M-CSF) glycoprotein is required for the survival, proliferation and differentiation of mononuclear phagocytes. M-CSF has been shown to be expressed by a variety of cell types and to be involved in placental and fetal development (see [5] for a review), as well as to play a role in atherogenesis [6]. M-CSF mediates its effects by binding to its high-affinity transmembrane receptor (M-CSFR), a protein tyrosine-kinase receptor encoded by the *c-fms* proto-oncogene (see [7] for a review). Cultured mouse mesangial cells and VSMCs from various species have been reported to synthesize constitutively and/or to secrete M-CSF following stimulation [8–12]. In addition, the levels of M-CSF mRNA in human arterial cells can be modulated in response to a variety of stimuli, including interleukin-1 α , tumour necrosis factor- α and phorbol esters [8,9]. Likewise, M-CSFR and its mRNA are expressed in mouse mesangial cells [10]. By contrast, *c-fms*

mRNA could not be detected in human saphenous vein smooth-muscle cells [8]. In an experimental rabbit model of arteriosclerosis, expression of *c-fms* was observed in VSMCs isolated from the altered intima, but not in those from the normal media [13].

These observations prompted us to study further *in vitro* the mechanisms whereby M-CSF might affect VSMC proliferation. Here we report that, in cultured VSMCs isolated from rat aorta: (i) biologically active M-CSF is synthesized and secreted, (ii) M-CSFR is expressed at both the protein and the mRNA level, and (iii) M-CSF behaves as a mitogen. These data indicate that M-CSF can affect the growth of VSMCs via autocrine/paracrine mechanisms, and suggest an important role for M-CSF in the pathophysiology of the arterial wall.

MATERIALS AND METHODS

Reagents

Cell culture materials and media were obtained from Costar and Life Technologies respectively. Lab-Tek chamber slides were from Nunc. Fetal-calf serum (FCS) was from Boehringer-Mannheim. Human recombinant M-CSF and neutralizing polyclonal rabbit anti-M-CSF were from Genzyme. Antibodies against myosin and smooth-muscle α -actin were from Novacastra, and rabbit polyclonal anti-c-Fms was from Santa Cruz. [³H]Thymidine (666 GBq/mmol) was from Amersham, and [α -³²P]dCTP (110 TBq/mmol) was from ICN. Hybond C extra nitrocellulose membranes were from Amersham.

The cDNA probes used for Northern blot analysis were: a 1.4 kb *Bam*HI fragment of the M-CSF gene [14]; a 1.3 kb *Pst*II

Abbreviations used: M-CSF, macrophage-colony-stimulating factor; M-CSFR, M-CSF receptor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; VSMC, vascular smooth-muscle cell; FCS, fetal-calf serum; DMEM, Dulbecco's modified Eagle's medium.

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fragment of the *v-fms* gene (pSM3 clone) [15]; a 1.5 kb *EcoRI* fragment of murine *junB* [16]; a 1.0 kb *PstI* insert of the *c-fos* gene purified from the p-fosI plasmid [17]; a 1.5 kb *ClaI/EcoRI* insert of the *c-myc* gene [18]; a 3.2 kb *EcoRI* insert of *egr-1* gene purified from plasmid pBluescript KS⁺ [19]; and a 2.2 kb insert of the mouse β -actin gene purified from the PAL41 plasmid [20]. The probes were labelled with [α -³²P]dCTP using the multiprime DNA labelling system (Amersham) as described by Feinberg and Vogelstein [21]. All other reagents were obtained from Sigma.

Cells and culture conditions

VSMCs were isolated from the thoracic aortae of 10-week-old Wistar Kyoto rats by the explant technique as described by Ross [22]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8 mM Hepes buffer, 100 units/ml penicillin, 100 μ g/ml streptomycin and 10% (v/v) FCS. Cells were subcultured weekly. In this study, cells between passages 4 and 12 were used. VSMCs grew in a characteristic 'hill-and-valley' pattern, and immunostaining procedures showed that cells were reactive to anti-myosin and anti- α -actin antibodies. For each experiment, VSMCs were cultured for 3 days in 10% FCS and made quiescent by serum deprivation for 2 days.

The L6 α 1 rat myogenic cell line and L929 cells, kindly donated by Dr. S. Leibovitch (CNRS, IGR, Villejuif, France), were cultured in DMEM plus 10% (v/v) FCS, as described by Borycki et al. [12]. The serum-free supernatant of L929 cells was used as a source of M-CSF [23–26].

Bioassay of M-CSF activity

M-CSF activity was measured as described by Gogusev et al. [27], by evaluating its ability to stimulate the growth of murine monocyte/macrophage colonies obtained from adult C57 black mouse bone marrow. In brief, colony formation was assayed on a double-layer agar culture system containing L-glutamine, penicillin/streptomycin and 20% (v/v) FCS in DMEM. Overlayers (0.5 ml) were composed of 0.33% (w/v) agar in the same medium. The serum-free supernatant of L929 cells, used as a potential source of M-CSF and thus as a positive control, and supernatants of VSMC cultures were added to the underlayers (containing 0.5% agar) at 25% (v/v) final concentration. Only large refractile colonies, morphologically identified as being mainly composed of macrophages and granulocytes, were counted on day 7. Colonies were identified as monocytes/macrophages or granulocytes using α -naphthyl acetate esterase and chloroacetate esterase staining respectively [28].

Immunocytochemistry procedures

For immunocytochemistry, VSMCs were cultured in Lab-Tek chamber slides for 3 days, washed in PBS, fixed in methanol/acetone (1:1, v/v) at 4 °C and incubated with the specific antibodies. Negative controls were performed using non-immune rabbit IgG instead of specific anti-Fms antibody. Immunoreactivity was revealed using the peroxidase-antiperoxidase-complex system following standard protocols.

DNA synthesis

The mitogenic effects of M-CSF, platelet-derived growth factor (PDGF), thrombin and L929 cell serum-free supernatant were determined by measuring [³H]thymidine incorporation into the DNA of VSMCs cultured in 24-well plates. Quiescent VSMCs were exposed for 24 h to either stimulus or vehicle in DMEM containing 1 μ M thymidine and 0.5 μ Ci/ml [³H]thymidine.

Thymidine incorporation was terminated by removing the medium and washing the cells twice with cold PBS (1 ml/well) prior to determination of radioactivity [29].

RNA extraction and Northern blot hybridization procedures

Total cellular RNA was isolated using the acid guanidinium/isothiocyanate method [30]. For Northern blot analysis, 10 μ g of total RNA was electrophoresed through a 1% (w/v) agarose/2.2 M formaldehyde gel and transferred to a Hybond C nitrocellulose membrane. The membranes were prehybridized at 42 °C for 6 h in a buffer consisting of 50% formamide, 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5 $\times$ Denhardt's solution, 0.1% SDS and 200 μ g/ml salmon sperm DNA. Hybridization reactions were carried out for 16–24 h at 42 °C in the same buffer with the appropriate ³²P-labelled cDNA probe (2 $\times$ 10⁶ c.p.m./ml). Membranes were washed to a stringency of 0.1 $\times$ SSC at 55 °C and exposed to Amersham Hyperfilm-MP using an intensifying screen. Quantitative evaluation of the transcripts was carried out by laser densitometric scanning (LKB 2202 Ultrascan) of the autoradiograms.

Statistical analysis

Values are given as means $\pm$ S.E.M. of (*n*) experiments. Each experiment involved one distinct culture; within each experiment, measurement of [³H]thymidine incorporation was carried out in duplicate (two wells). Unless otherwise stated, tests of significance were performed using the unpaired Student's *t* test.

RESULTS

It has been reported that immunoreactive M-CSF is constitutively produced by cultured human VSMCs, as well as by cultured mouse mesangial cells [9,10]. Therefore, by using a bioassay which measured the M-CSF-induced colony formation of murine bone marrow cells [25], we first determined whether supernatants from rat aortic VSMC cultures contained biologically active M-CSF. The numbers of large and refractile murine bone marrow colonies identified as macrophages/monocytes, induced by 25% (v/v) supernatants of VSMCs cultured for 24 h, are reported in Table 1. The culture medium, in either the presence or the absence of serum, did not promote colony formation, whereas

Table 1 Secretion of M-CSF by VSMCs

Colony formation of murine bone marrow cells was measured in the presence of supernatants from VSMCs or L929 cells, as described in the Materials and methods section. Serum-free supernatant from L929 cell cultures and recombinant M-CSF (500 units/ml) were used as positive controls. Anti-M-CSF antibody was used at 1 μ g/ml. L929 cells and VSMCs were cultured for 3–4 days in DMEM containing 10% (v/v) FCS and rendered quiescent by serum deprivation for 2 days in DMEM. The supernatants thus obtained (L929 sup. and VSMC sup.) were used at 25% (v/v) in the bioassay. Values are means $\pm$ S.E.M. of triplicate assays of bone marrow cell colony formation. ND, not detectable.

Treatment	Murine colonies (5 $\times$ 10 ⁴ marrow cells)	
	Monocytes/macrophages	Granulocytes
DMEM	ND	ND
DMEM + 10% FCS	1 $\pm$ 1	1 $\pm$ 1
DMEM + M-CSF	205 $\pm$ 12	5 $\pm$ 1
L929 sup.	66 $\pm$ 15	5 $\pm$ 1
L929 sup. + anti-M-CSF	16 $\pm$ 5	ND
VSMC sup.	21 $\pm$ 6	8 $\pm$ 1
VSMC sup. + anti-M-CSF	7 $\pm$ 2	ND

Figure 1 M-CSF, M-CSFR (*c-fms*) and β -actin gene expression in VSMCs and L6 α 1 cells

Total cellular RNA was extracted from unstimulated (control) or 10%-FCS-stimulated VSMCs (or L6 α 1 cells) for the indicated times and was hybridized with specific 32 P-labelled cDNA probes for M-CSF, M-CSFR (*c-fms*) and β -actin under the experimental conditions described in the Materials and methods section. The results shown are representative of three experiments.

the positive controls, i.e. serum-free L929 cell supernatant or M-CSF added directly to the bone marrow agar cultures, did so (Table 1). The results also show: (i) the low numbers of granulocyte colonies compared with monocyte/macrophage colonies obtained using L929 cell supernatant, and (ii) that the induction of monocyte/macrophage colonies could be decreased by the addition of anti-M-CSF antibody (Table 1). These findings indicated that, under the experimental conditions used, serum-free supernatant from L929 cells might well be considered as a source of M-CSF. On the other hand, serum-free supernatant from VSMCs induced a significant number of monocyte/macrophage colonies which could be specifically decreased by the addition of anti-M-CSF antibody (Table 1).

In another series of experiments, using Northern blot analysis, VSMCs were demonstrated to express an ~ 4.6 kb M-CSF transcript (Figure 1). In a time-course experiment, following VSMC stimulation by serum, the level of M-CSF mRNA reached a maximum after 1–4 h and was still above the control level after 24 h (Figure 1). Scanning of the autoradiograms obtained from several experiments confirmed these changes (results not shown). In parallel, we observed that serum-stimulated L6 α 1 rat myogenic cells (taken as a reference for M-CSF gene expression [31]) also expressed the specific mRNA (Figure 1).

In addition, we determined whether rat aortic VSMCs expressed the M-CSFR, as has been demonstrated for rabbit and human VSMCs under specific conditions [13,32]. In a first series of experiments, the presence of M-CSFR in VSMCs was clearly

Figure 2 Immunocytochemical detection of M-CSFR expression

M-CSFR, the protein receptor encoded by *c-fms*, was detected by immunocytochemistry in VSMCs, by using rabbit polyclonal anti-c-Fms antibody (upper panel). Negative staining obtained using non-immune serum is shown for comparison (lower panel). Detailed procedures were as described in the Materials and methods section. The photomicrographs (representative of two experiments) are shown at a magnification of $\times 200$ (upper panel) and $\times 300$ (lower panel).

revealed by immunocytochemistry using a polyclonal anti-c-Fms antibody (Figure 2, upper panel). Used as positive controls [12], L6 α 1 cells exhibited similar immunolabelling when incubated with the specific anti-c-Fms antibody (results not shown). In a control experiment using non-immune rabbit serum, no specific immunostaining was observed (Figure 2, lower panel). In a second series of experiments, using the same RNA preparations as those used for the detection of M-CSF transcripts, Northern blot analysis unambiguously revealed the presence of an ~ 4.1 kb *c-fms* mRNA in VSMCs as well as in L6 α 1 myogenic cells used as a positive control (Figure 1). Figure 1 also shows that, in a time-course experiment, the level of the *c-fms* transcript, like that of β -actin, did not vary over 24 h.

Since M-CSF has been shown to be a mitogen in several cell types [5], we analysed whether this growth factor could promote DNA synthesis in rat aortic VSMCs. After treatment of quiescent VSMCs with serum-free supernatant from L929 cells as a source of M-CSF, we observed that the incorporation of [3 H]thymidine increased in a concentration-dependent manner (Table 2a). This effect could be decreased by the addition of anti-M-CSF antibody, whereas the addition of non-immune IgG was without effect (Table 2a). The incorporation of [3 H]thymidine in VSMCs could also be induced by recombinant human M-CSF

Table 2 Mitogenic effects of L929 cell supernatant on VSMCs

[³H]Thymidine incorporation into VSMCs was measured following the stimulation of quiescent cells by serum-free L929 cell supernatant at various dilutions (a) or by recombinant human M-CSF at various concentrations (b), under the conditions detailed in the Materials and methods section. Where indicated, L929 cell supernatant was preincubated for 1 h prior to stimulation with 1 µg/ml anti-M-CSF antibody or non-immune IgG. Values are means ± S.E.M. of (n) experiments. Statistical analysis: **P* < 0.05; ***P* < 0.01 compared with basal (unstimulated cells); †*P* < 0.05 compared with 1:4 dilution.

Addition	[³ H]Thymidine incorporation (c.p.m./10 ⁵ cells)
(a)	
DMEM (basal)	167 ± 28 (8)
L929 supernatant diluted:	
1:16	185 ± 29* (8)
1:8	240 ± 47** (8)
1:4	367 ± 61** (8)
1:4 + anti-M-CSF antibody	238 ± 30† (8)
1:4 + non-immune IgG	387 ± 51** (8)
(b)	
DMEM (basal)	94 ± 4 (7)
M-CSF (100 units/ml)	117 ± 4* (6)
M-CSF (500 units/ml)	148 ± 10** (5)

Table 3 Combined addition of M-CSF with thrombin, PDGF or bFGF synergistically increases DNA synthesis in VSMCs

[³H]Thymidine incorporation into VSMCs was measured following the stimulation of quiescent cells by thrombin (0.5 unit/ml), PDGF (5 ng/ml) or bFGF (20 ng/ml), alone or in combination with recombinant M-CSF (500 units/ml). Values are means ± S.E.M. (n = 6). Values obtained in the presence of M-CSF are compared with those obtained in the absence of M-CSF: **P* < 0.01.

Addition	[³ H]Thymidine incorporation (c.p.m./10 ⁵ cells)	
	— M-CSF	+ M-CSF
DMEM (basal)	88 ± 7	149 ± 9*
Thrombin	1101 ± 92	1691 ± 202*
PDGF	1379 ± 145	2163 ± 243*
bFGF	1418 ± 125	2085 ± 207*

Table 4 Mitogenic effect of endogenous M-CSF

VSMCs were cultured for 3 days in 10% (v/v) FCS-containing medium and rendered quiescent by serum deprivation for 2 days. After removal of supernatant, fresh serum-free medium was added and VSMCs were stimulated by DMEM or serum-free supernatant from VSMCs (VSMC sup.), in the absence or the presence of anti-M-CSF antibody. The serum-free supernatant was preincubated for 1 h prior to stimulation with anti-M-CSF antibody (1 µg/ml). [³H]Thymidine incorporation was then measured as detailed in the Materials and methods section. Values are means ± S.E.M. of (n) experiments. Statistical analysis: **P* < 0.05, ***P* < 0.001 compared with DMEM; †*P* < 0.001 compared with VSMC sup.

Addition	[³ H]Thymidine incorporation (c.p.m./10 ⁵ cells)
DMEM (basal)	120 ± 10 (4)
DMEM + anti-M-CSF	95 ± 12* (6)
VSMC sup.	265 ± 24** (5)
VSMC sup. + anti-M-CSF	172 ± 29† (5)

in a concentration-dependent manner (Table 2b). Moreover, when M-CSF was added in concert with thrombin, PDGF or basic fibroblast growth factor (bFGF) to quiescent VSMCs, a synergistic increase in DNA synthesis was observed (Table 3).

Figure 3 Expression of *junB*, *c-myc*, *c-fos*, *egr-1* and β -actin genes in VSMCs

The kinetics of expression of the *junB*, *c-myc*, *c-fos*, *egr-1* and β -actin genes were studied by Northern blot analysis following the stimulation of VSMCs by 25% (v/v) L929 cell supernatant (sup) for the times indicated.

Results (not shown) similar to those in Table 3 were obtained by replacing M-CSF by 25% (v/v) serum-free L929 cell supernatant.

Since biologically active M-CSF has been shown to be released into the culture medium by VSMCs (Table 1), the possibility was investigated that such endogenous M-CSF might exert its mitogenicity on VSMCs through autocrine/paracrine mechanisms. Serum-free supernatant from VSMCs was prepared as described above and added to quiescent VSMCs in serum-free medium in the absence or presence of anti-M-CSF antibody, and the incorporation of [³H]thymidine was measured. The results clearly indicated that the VSMC supernatant enhanced [³H]thymidine incorporation, and that this effect could be decreased significantly by anti-M-CSF antibody (Table 4).

To confirm the mitogenic action of M-CSF on VSMCs, the effect of L929 cell supernatant on the expression of proliferation-associated genes was examined by Northern blot analysis. Figure 3 represents a time-course experiment in which the addition of serum-free supernatant from L929 cells to quiescent VSMCs modulated the expression of the *junB*, *c-myc*, *c-fos* and *egr-1* proto-oncogenes. Figure 3 also shows that, under the same

conditions, no changes were observed in the level of expression of the β -actin gene.

DISCUSSION

The regulation of VSMC proliferation in the arterial wall is considered to be central to the pathogenesis of atherosclerosis [1]. In this respect, a number of studies have provided compelling evidence for the involvement of M-CSF in the atherosclerotic process as well as in arterial hypertension [6,8,9,13,23,32,33]. Therefore it was of interest to investigate the possible influence of M-CSF on VSMC growth.

In the present study, we have demonstrated that the supernatant of cultured VSMCs isolated from rat aorta exhibited a monocyte/macrophage-colony-forming activity, probably due to M-CSF (Table 1). We also demonstrated that VSMCs constitutively express M-CSF mRNA and that this expression could be increased by serum (Figure 1). These findings strongly suggest that cultured VSMCs isolated from rat aorta synthesize and secrete biologically active M-CSF. In addition, the presence of both M-CSFR mRNA and protein in VSMCs was demonstrated by Northern blot analysis and immunocytochemical procedures respectively (Figures 1 and 2).

Since cultured VSMCs co-expressed M-CSF and its receptor, it was interesting to examine whether M-CSF might affect the growth of VSMCs through autocrine/paracrine mechanisms. The present results clearly indicate that both serum-free supernatant from L929 cells (used as a source of M-CSF) and recombinant M-CSF could induce [3 H]thymidine incorporation in a concentration-dependent manner, and that this effect could be abrogated by the concomitant addition of anti-M-CSF antibody (Table 2). The results also show that a synergistic effect on [3 H]thymidine incorporation was observed following the combined addition of M-CSF with thrombin, PDGF or bFGF (Table 3). In this respect, it is interesting to note that PDGF has been reported to induce the expression of *c-fms* in VSMCs of tissues that normally do not express this gene [34]. Likewise, thrombin has been shown to enhance M-CSF-induced mitogenesis [35]. Moreover, the finding that the addition of serum-free L929 cell supernatant to VSMCs could trigger and/or regulate the expression of mitogen-inducible genes, such as *c-fos*, *c-myc*, *junB* and *egr-1* (Figure 3) [36], is consistent with the concept of M-CSF being a growth factor for VSMCs. The simultaneous expression of M-CSF and its receptor in VSMCs, along with the finding that endogenous M-CSF elicits DNA synthesis in VSMCs (Table 4), strongly suggest the involvement of autocrine/paracrine mechanisms in the growth control of this cell type by M-CSF.

Expression of *c-fms* has been demonstrated in aortic smooth-muscle cells isolated from an experimental rabbit model of atherosclerosis, but not in cells isolated from normal media [13]. Human smooth-muscle cells isolated from saphenous veins did not express *c-fms* mRNA, even after treatment with lipopolysaccharide or interleukin-1 α [8]. The co-expression of M-CSF and M-CSFR has been described in mouse mesangial cells [10]. However, in these experiments the exogenous addition of M-CSF (up to 2000 units/ml) did not stimulate [3 H]thymidine incorporation. Our results therefore indicate that cultured VSMCs from rat aorta could behave, in some respect, like cells isolated from atherosclerotic lesions, and thus could be considered as a model for investigating the mechanisms whereby M-CSF modulates VSMC growth.

One may hypothesize, therefore, that both VSMCs and macrophages, two cell types that synthesize and secrete M-CSF and express M-CSFR, play a pivotal role in the progression of

atherosclerotic lesions. Under such circumstances, M-CSF, acting as a growth factor on these cells, is likely to be involved in the development of vascular alterations. In this context, the expression of M-CSF and/or M-CSFR is increased by pro-inflammatory mediators [32,37] and can also be stimulated by low-density lipoprotein [38]. Other studies have shown that lipoproteins together with angiotensin II or epidermal growth factor synergistically stimulated the growth-related metabolism of VSMC [39,40]. Thus M-CSF might act in concert with other growth factors resulting in synergy of their growth effects, thereby accelerating atherosclerotic progression.

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