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Expression and secretion of *gro*/MGSA by stimulated human endothelial cells

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Melanoma growth stimulatory activity factor (MGSA) is a polypeptide which was initially isolated from Hs294 human melanoma cells. Its sequence is identical to the deduced amino acid sequence of the human *gro* cDNA, isolated from a human tumor cell line. MGSA stimulates the proliferation of malignant melanoma cells, but its function for normal cells has not been defined. Here we report that human umbilical vein endothelial cells are capable of synthesizing and secreting MGSA. The expression and secretion of MGSA are strongly induced by factors often involved in inflammation such as IL-1, TNF, LPS and thrombin. The induction of MGSA mRNA is dose and time dependent and is independent of new protein synthesis. This stimulation could be mimicked by TPA, suggesting that the action could be mediated through activation of protein kinase C. Furthermore, addition of MGSA to the endothelial cell cultures induces *gro*/MGSA gene expression, implying that an autocrine mechanism exists. Our data suggest that the protein encoded by *gro*/MGSA mRNA may play a role in inflammation and exert its effects on endothelial cells in an autocrine fashion.

Key words: *gro*/MGSA/cytokines/thrombin/inflammation/autocrine/endothelial cells

Introduction

A recently isolated factor that stimulates the proliferation of melanoma cells has been called 'melanoma growth stimulatory activity' (MGSA) (Richmond and Thomas, 1986). cDNA characterization (Richmond *et al.*, 1988) established that the deduced amino acid sequence for human MGSA is identical to the deduced amino acid sequence of the human '*gro*' cDNA (Anisowicz *et al.*, 1988). The *gro* mRNA was initially detected as an mRNA that was elevated in several tumor cell lines and could be transiently induced in some normal cells by serum (Anisowicz *et al.*, 1987). No function has been assigned yet to the protein encoded by the *gro* cDNA. Northern analysis using the human MGSA cDNA probe showed that the corresponding mRNA was expressed in various tumor cell lines and in non-transformed fibroblasts and mammary epithelial cells and that the expression of MGSA is thus not at all restricted to melanoma cells (Anisowicz *et al.*, 1987; Richmond *et al.*, 1988). These data thus suggest that MGSA may be a normal regulator of cell proliferation for a variety of cell types. The amino acid sequence deduced from the cDNAs revealed that the precursor for MGSA is 107 residues long and that the mature factor is a polypeptide of 73 amino acids (Anisowicz *et al.*, 1987; Richmond *et al.*, 1988). The elucidation of the sequence also clearly showed that human *gro*/MGSA is structurally related to various other factors, for which the functions are generally ill-defined or still have to be established (Anisowicz *et al.*, 1987; Richmond *et al.*, 1988). The two best-known members of the family are platelet factor-4 and platelet basic protein, which is proteolytically processed into β-thromboglobulin and connective tissue activating peptide-3 (CTAP III). Platelet factor 4, β-thromboglobulin and CTAP-III are stored in platelets and released into the bloodstream upon thrombin stimulation of the platelets (Deuel *et al.*, 1977; Begg *et al.*, 1978; Castor *et al.*, 1983; Holt *et al.*, 1986; Poncz *et al.*, 1987).

Endothelial cells form the luminal surface of the vascular system and constitute the metabolic interphase between the blood and the vascular vessel wall and tissues. Endothelial cells are thus located in an important interphase and play a role in a wide range of vital physiological functions, such as inflammation, hemostasis and wound healing. Recent studies have indicated that endothelial cells can be induced to secrete a variety of factors that are involved in cell proliferation and immunomodulation and that they have receptors for many factors that play a role in these processes (Barrett *et al.*, 1984; Collins *et al.*, 1985; Stern *et al.*, 1985; Broudy *et al.*, 1986; Daniel *et al.*, 1986; Libby *et al.*, 1986; Munker *et al.*, 1986; Jirik *et al.*, 1987). The storage of factors related to MGSA in platelets and their release in close proximity to the endothelial cells and the binding of MGSA to heparin, a constituent of the basement membrane on which the endothelial cells are located, lead us to evaluate the expression of MGSA in human umbilical vein endothelial cells. This study demonstrates that these endothelial cells are capable of synthesizing the MGSA mRNA and protein and that the expression of this mRNA can be induced by the MGSA protein itself, thus implying the presence of receptors on these cells. The expression of the mRNA and the secretion of the protein are strongly induced by interleukin-1 (IL-1), tumor necrosis factor (TNF), lipopolysaccharide (LPS) and thrombin. These observations indicate that secretion of the MGSA protein is induced by factors that are often involved in inflammation. The protein encoded by the *gro*/MGSA mRNA may thus play a role in inflammation and exert its effects on the endothelial cells in an autocrine fashion.

Results

Auto-induction of gro/MGSA mRNA in human endothelial cells

The initial experiments were designed to determine whether the *gro*/MGSA mRNA is expressed in human endothelial cells and whether these cells are responsive to the MGSA protein. Northern hybridization showed that a low level of MGSA mRNA can be detected in untreated endothelial cells (Figure 1A, 0 h lane). The size of the mRNA is ~1.2 kb,

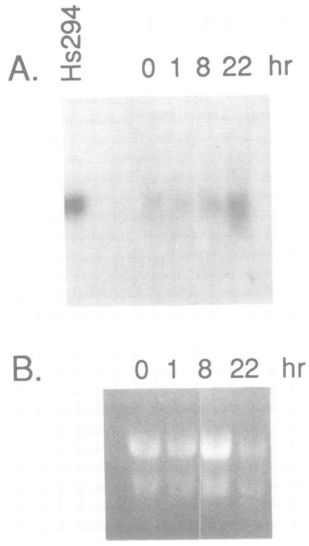

Fig. 1. (**A**) Exogenous MGSA administration increases the levels of *gro*/MGSA mRNA in cultured human umbilical vein endothelial cells. Endothelial cells were incubated with MGSA (3 ng/ml) in serum-free medium. Total cytoplasmic RNA was isolated at appropriate times as indicated and analyzed by Northern hybridization. RNA from Hs294 cells was used as a positive control for Northern hybridization. **B**) Ethidium bromide staining of the total RNA applied to each lane. The amount of RNA in the last lane is less than in the other lanes.

a value identical to the mRNA originally detected in the Hs294 melanoma cells (Figure 1A) (Richmond *et al.*, 1988). To evaluate whether the endothelial cells are responsive to exogenous MGSA and thus contain receptors for this factor, we relied on the ability of *gro*/MGSA to induce its own expression. This auto-induction mechanism has previously been demonstrated for MGSA in the responsive Hs294 cells (Richmond *et al.*, 1988) and has also been described for several other growth factors, such as TGF-α (Coffey *et al.*, 1987), TGF-β (Van Obberghen-Schilling *et al.*, 1988), IL-1 (Warner *et al.*, 1987) and PDGF (Paulsson *et al.*, 1987). Direct demonstration of the receptor using radio-iodination of the ligand followed by cross-linking, or evaluation of the mitogenic effect of MGSA on endothelial cells was not possible due to the minute quantities (nanograms) of the factor available to us. Human endothelial cells were incubated with MGSA (3 ng/ml) in serum-free medium. Total cytoplasmic RNA was isolated at appropriate times and analyzed by Northern hybridizations. Figure 1A shows that *gro*/MGSA mRNA levels were increased after 8 h and much more elevated at 22 h. The increase of the MGSA mRNA hybridization signal is underestimated in Figure 1A due to the smaller quantity of RNA loaded in the 22 h lane (Figure 1B). The induction of MGSA mRNA by the MGSA protein itself is similar to what we have previously described for the Hs294 melanoma cells (Richmond *et al.*, 1988). These data indicate that endothelial cells are responsive to exogenously administered MGSA, implying the existence of receptors for this factor on the cell surface.

Induction of gro/MGSA expression in endothelial cells

Endothelial cells *in vivo* can be exposed to a variety of factors that are involved in cell proliferation and immunomodulation, especially at sites of inflammation and neovascularization. We therefore evaluated whether various factors can influence

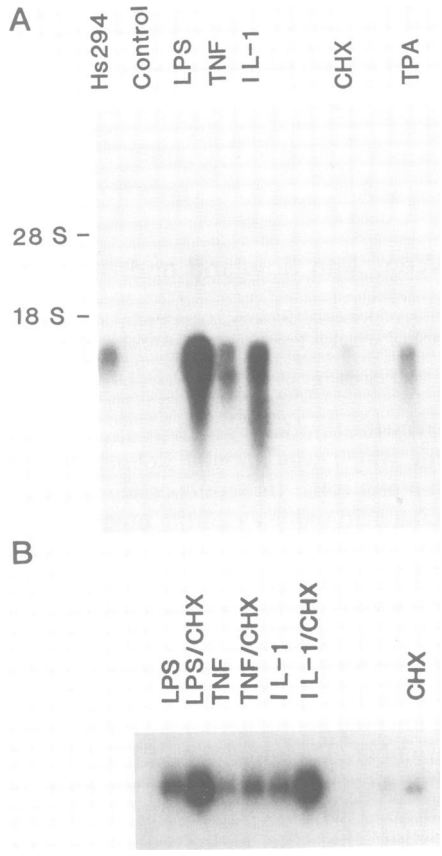

Fig. 2. (**A**) Induction of *gro*/MGSA expression in endothelial cells. Confluent endothelial cells were treated wtih IL-1β (5 U/ml), TNF-α (100 ng/ml), LPS (10 μg/ml), TPA (100 ng/ml) or cycloheximide (CHX, 10 μg/ml) respectively for 3 h at 37°C. Total cytoplasmic RNA was isolated, fractionated by agarose gel electrophoresis (10 μg/each lane) and analyzed by Northern hybridization. Control, no treatment. (**B**) Effect of cycloheximide on the induction of MGSA mRNA. Endothelial cells were incubated with LPS, TNF or IL-1 in the presence or absence of cycloheximide (10 μg/ml) as indicated. Total cytoplasmic RNA was analyzed by Northern hybridization.

the expression of *gro*/MGSA in endothelial cells. Confluent human umbilical vein endothelial cells were incubated with IL-1β (5 U/ml), TNF-α (100 ng/ml) or LPS (10 μg/ml). Total cytoplasmic RNA was isolated 3 h after administration of the factor and analyzed by Northern hybridization for the presence of *gro*/MGSA mRNA. As shown in Figure 2A, the steady state level of *gro*/MGSA mRNA was dramatically increased by IL-1β, LPS and TNF-α treatment, but was barely detectable in the untreated cells. Besides the 1.2 kb mRNA, there was a low level of a 0.9 kb mRNA, which could correspond to an mRNA with altered sites of transcriptional initiation or polyadenylation. The levels of MGSA mRNA observed after IL-1 or LPS treatment were significantly higher than in the Hs294 melanoma cells. We also tested IL-1α, which binds to the same receptor as IL-1β, and lymphotoxin (TNF-β), which shares with TNF-α the same receptor, and found the same level of MGSA mRNA induction (data not shown). In addition, TPA, which activates the cellular protein kinase C (Kikkawa and Nishizuka, 1986), also had a mild effect on the MGSA expression (Figure 2A). Several other cytokines were also tested for their effect on endothelial *gro*/MGSA mRNA levels. Neither IFN-γ (100 ng/ml) nor IL-6 (10 U/ml) or TGF-β (20 ng/ml) affected the expression of MGSA when added alone or

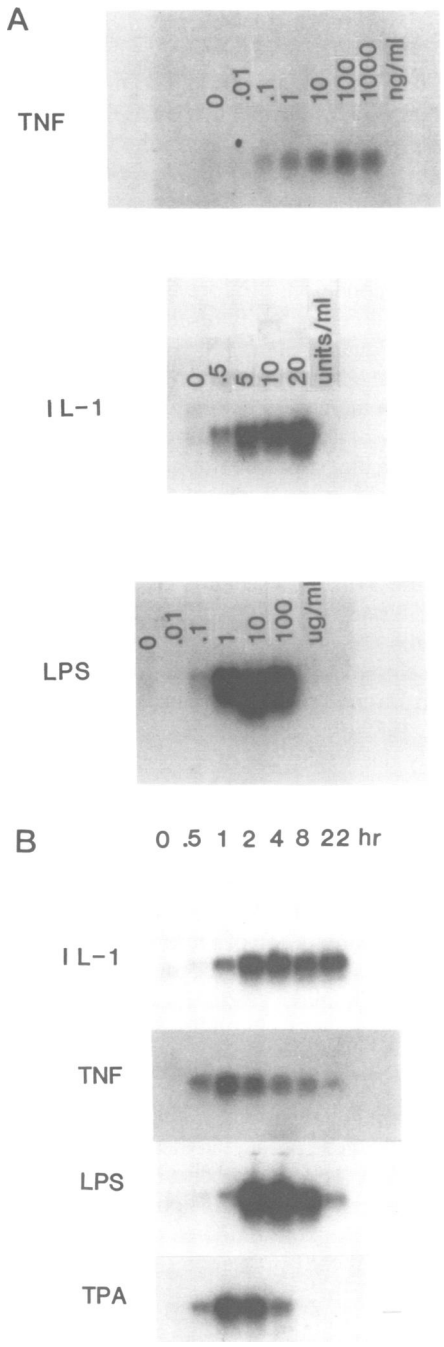

Fig. 3. (**A**) Dose response of *gro*/MGSA mRNA induction in endothelial cells. Confluent endothelial cells were incubated for 4 h with TNF, IL-1 or LPS at increasing concentrations of the inducers as indicated. Total cytoplasmic RNA was isolated and analyzed by Northern hybridization. (**B**) Kinetics of MGSA induction. Confluent endothelial cells were treated with IL-1 (5 U/ml), TNF (100 ng/ml), LPS (10 μg/ml) or TPA (100 ng/ml) at 37°C. Total cytoplasmic RNA was isolated at the indicated times and analyzed by Northern hybridization.

together with IL-1, TNF or LPS (data not shown). While this manuscript was in preparation, Anisowicz *et al.* (1988) reported the induction of the *gro*/MGSA mRNA in foreskin fibroblasts by TPA and IL-1.

Because TNF-α and LPS both can induce IL-1 in endothelial cells (Libby *et al.*, 1986), it would be possible that the effect by TNF and LPS was through IL-1 as the mediator.

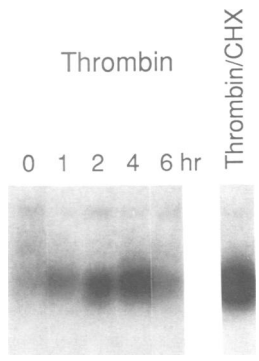

Fig. 4. Effect of thrombin on *gro*/MGSA expression. Confluent endothelial cells were placed overnight in serum-free medium containing insulin (1 μg/ml), transferrin (5 μg/ml) and BSA (0.5 mg/ml). Cells were then refed with fresh medium and treated with thrombin (3 U/ml). Total cytoplasmic RNA was isolated at the indicated times and analyzed by Northern hybridization. Thrombin/CHX, cells were treated with thrombin (3 U/ml) plus cycloheximide (10 μg/ml) at 37°C for 4 h.

Treatment with cycloheximide, an inhibitor of translation, was tested to determine if *gro*/MGSA expression was dependent upon new protein synthesis. Confluent cells were treated for 4 h with IL-1β, TNF or LPS in the presence or absence of cycloheximide (10 μg/ml). We found that cycloheximide did not suppress the MGSA expression; instead it increased the level of MGSA mRNA at least 2-fold, when it was added together with the inducer (Figure 2B). Treatment with cycloheximide alone showed a minor increase of *gro*/MGSA mRNA, presumably due to a stabilization of the mRNA or inhibition of a protein that down-regulates *gro*/MGSA. Previous cDNA characterization has revealed that the 3′-untranslated sequence of the *gro*/MGSA cDNA predicts AU-rich sequences that mediate a fast mRNA degradation (Anisowicz *et al.*, 1987; Richmond *et al.*, 1988). The increase of *gro*/MGSA mRNA level by cycloheximide has previously been demonstrated in fibroblasts and melanoma cells (Anisowicz *et al.*, 1987; Richmond *et al.*, 1988).

Induction of gro/MGSA expression is dose and time dependent

Dose−response experiments indicated that induction of *gro*/MGSA expression was dependent on the concentration of the factors added. Elevated levels of MGSA mRNA could be detected when cells were treated for 4 h with as little as 0.1 ng/ml of TNF-α, 0.5 U/ml of IL-1β or 0.1 μg/ml of LPS, increasing steadily with increasing doses up to a maximum level of expression (Figure 3A). The increase in MGSA mRNA was also observed in a time-dependent manner after initiation of the treatment. This increase was first detectable at 0.5 h for TNF-α and TPA, and at 1 h for IL-1 and LPS. Maximum level was reached between 1 and 4 h after the treatment. MGSA expression subsequently declined toward near basal levels (Figure 3B).

Thrombin induction of gro/MGSA expression

Recent studies indicated that endothelial cells have cell surface receptors for thrombin (Low and Cunningham, 1982), which is capable of inducing IL-1 and PDGF secretion from cultured endothelial cells (Stern *et al.*, 1985;

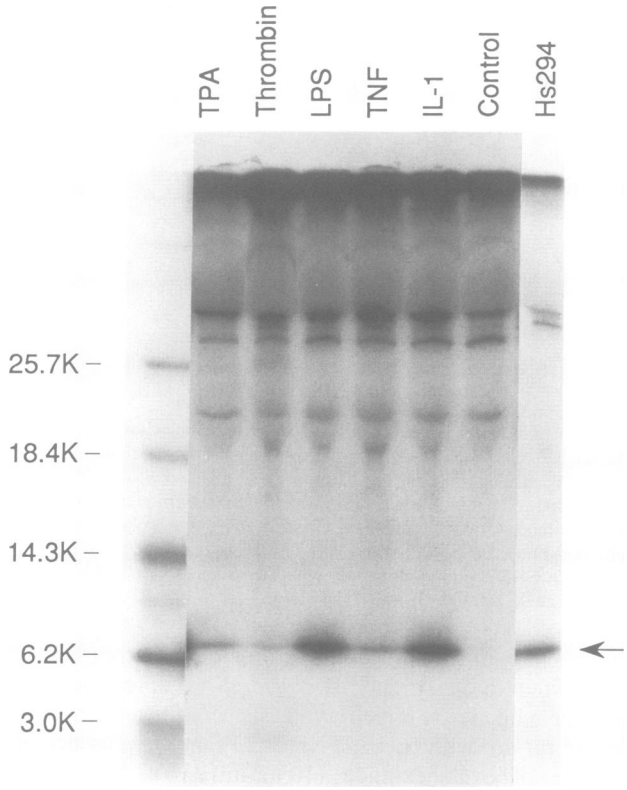

Fig. 5. Secretion of *gro*/MGSA by endothelial cells. Endothelial cells were washed with PBS and refed with 1.5 ml of methionine- and cysteine-free medium containing the inducers as indicated. After 1 h, [^{35}S]methionine (150 μCi) and [^{35}S]cysteine (100 μCi) were added and cells were incubated at 37°C overnight. The medium was collected. The secreted MGSA was precipitated by rabbit anti-MGSA peptide antiserum and analyzed by tricine–SDS–PAGE as described in Materials and methods. Control, no treatment. Hs294, MGSA precipitated from Hs294 cell medium.

Daniel *et al.*, 1986). In addition, fibrin deposition is often associated with endothelial cell proliferation, which could at least in part be due to the action of thrombin. We therefore examined the effect of thrombin on MGSA expression by endothelial cells. In contrast to previous studies with cytokines and LPS, treatments with thrombin were carried out in serum-free medium to prevent inactivation of thrombin by thrombin inhibitors normally present in serum (Furie and Furie, 1988). Confluent monolayers of human endothelial cells were grown in serum-free medium overnight and the medium was then replaced with fresh serum-free medium containing thrombin (3 U/ml). Total cytoplasmic RNA was isolated at different times and analyzed for induction of MGSA mRNA. As shown in Figure 4, MGSA mRNA levels peaked at 4 h in response to thrombin treatment and declined rapidly at 6 h. Again, cycloheximide increased the MGSA level ~2-fold when used together with thrombin. Thrombin-induced MGSA expression could not be attributed to the presence of trace amounts of L-1, TNF or LPS in the preparation since pretreatment of thrombin with the specific inhibitors diisopropyl fluorophosphate (DFP) or hirudin blocked its ability to induce *gro*/MGSA expression (data not shown).

Secretion of gro/MGSA by stimulated endothelial cells

The experiments shown above evaluated the mRNA levels. In order to characterize and quantitate the corresponding MGSA protein, we developed an MGSA-specific antiserum raised against a C-terminal peptide of the factor. Confluent endothelial cells were metabolically labeled with [^{35}S]-methionine and [^{35}S]cysteine in the presence or absence of the inducing reagents. The culture medium was collected, immunoprecipitated and analyzed by tricine–SDS–PAGE under reducing conditions. Figure 5 shows that treatment of the endothelial cell cultures resulted in the secretion of easily detectable quantities of the MGSA protein. Both IL-1 and LPS were the best inducers for MGSA secretion, which is consistent with the results of the mRNA levels. Untreated endothelial cells did not secrete any *gro*/MGSA that was detectable under the autoradiographic exposure conditions used for the treated cells. A control immunoprecipitation of an induced culture using the normal rabbit serum resulted in a non-specific pattern similar to the uninduced control shown in Figure 5 (data not shown). The immunoprecipitated MGSA co-migrated with the MGSA made by Hs294 melanoma cells. The apparent mol. wt of 8 kd is consistent with the value predicted from the amino acid sequence, but is different from the previously reported value (Richmond and Thomas, 1985; Richmond *et al.*, 1988). This difference is presumably due to the use of a different gel system and mol. wt standards.

Discussion

MGSA was originally identified as a factor secreted into the medium by the human Hs294 melanoma cells that is able to stimulate the proliferation of these same cells and is thus a candidate autocrine growth factor for melanoma cells (Richmond and Thomas, 1986). Its amino acid sequence shows it to be identical to the polypeptide encoded by the human *gro* gene (Anisowicz *et al.*, 1987; Richmond *et al.*, 1988). The expression of the *gro* gene is induced in several fibroblast and epithelial cell lines, but no function has been assigned yet to the gene product in these systems (Anisowicz *et al.*, 1987, 1988). The gene product of the mouse KC gene, which is induced in various cell lines following serum or PDGF treatment, has a high degree of sequence similarity to human *gro*/MGSA (Oquendo *et al.*, 1989) and is presumably the murine homolog of the hamster *gro* (Anisowicz *et al.*, 1987). However, it is as yet unclear that the human *gro*/MGSA represents the homolog of the murine KC and hamster *gro*, due to some striking amino acid sequence differences and to the existence of several, structurally closely related genes in the human genome (D.Wen and R.Derynck, unpublished). The proteins encoded by these genes belong to a larger family of secreted factors (Anisowicz *et al.*, 1987; Richmond *et al.*, 1988; Oquendo *et al.*, 1989).

We have shown in this report that the human *gro*/MGSA product is synthesized by human umbilical vein endothelial cells and that its synthesis is highly inducible by IL-1, TNF-α, LPS and thrombin but not by interferon-γ, IL-6 and TGF-β. Simultaneous incubation of these inducers with cycloheximide resulted in even higher levels of *gro*/MGSA mRNA. In addition, the stimulation was mimicked by TPA,

indicating that the induction could be mediated in part through activation of protein kinase C. The finding that the capacity to induce *gro*/MGSA is shared by both IL-1 and TNF is not surprising, since both cytokines exert already multiple overlapping actions (Pober *et al.*, 1986; Harrison and Campbell, 1988). Inhibition of the proteolytic activity of thrombin by hirudin or DFP treatment (Awbrey *et al.*, 1979; Low and Cunningham, 1982) resulted in a lack of *gro*/MGSA induction in the endothelial cells, indicating an involvement of the protease activity in this action of thrombin. It has similarly been shown in fibroblasts that the mitogenic effect of thrombin depends on its activity as protease (Carney *et al.*, 1986; Cunningham *et al.*, 1985; Magnaldo *et al.*, 1988). We did not test the effect of PDGF, an inducer of the KC gene (Cochran *et al.*, 1983), because of the absence of specific receptors on endothelial cells (L.T.Williams, personal communication).

Exposure of mononuclear phagocytes to bacterial LPS results in the synthesis and secretion of IL-1 and TNF (Oppenheim *et al.*, 1986; Sherry and Cerami, 1988), while IL-1 is also synthesized by endothelial cells upon exposure to LPS (Libby *et al.*, 1986) or thrombin (Stern *et al.*, 1985). The exertion of the biological activities and the synthesis of these factors, which induce the synthesis of MGSA, are thus closely connected. IL-1 and TNF, the major cytokines secreted by stimulated mononuclear phagocytes and involved in the inflammatory response, elaborate a variety of effects on endothelial cells that result in 'endothelial activation' (Willms-Kretschmer *et al.*, 1967; Pober *et al.*, 1986; Cotran, 1987). The net result is the induction of an endothelial phenotype that is pro-inflammatory and markedly adhesive for leukocytes, presumably due to a rapid increase in cell surface expression of the adhesion molecules ICAM-1 and ELAM-1 (Bevilacqua *et al.*, 1987; Rothlein *et al.*, 1988). This endothelial activation manifests itself also in the induction of a procoagulant activity in endothelial cells (Chesterman, 1988) that coincides with an increased production of tissue factor (Nawroth *et al.*, 1986), release of plasminogen activator inhibitor-1 and decrease of tissue type plasminogen activator (Schleef *et al.*, 1988). This activation also results in some morphological changes and hypertrophy of the endothelial cells (Cotran, 1987). Our findings thus suggest that the protein encoded by the *gro*/MGSA gene may participate in this endothelial activation. It is likely that at least some of the activities of this factor are exerted in an autocrine fashion, since the autoinduction of MGSA mRNA in endothelial cells implies the presence of MGSA receptors on these cells.

At present it is not known what functions the recently discovered MGSA may have, except for its mitogenic activity on Hs294 melanoma cells (Richmond and Thomas, 1986). This suggests that this factor may be mitogenic for the endothelial cells as well. A potential function as autocrine growth factor for these cells could be suggested, especially since serum, PDGF and EGF have been shown to induce the closely related KC mRNA in several cell lines (Cochran *et al.*, 1983; Takehara *et al.*, 1987). However, sufficient quantities of this factor will be needed before a mitogenic activity on endothelial cells or any other action can be verified and established. In any event, the secretion and autoinduction of this polypeptide by endothelial cells and its strong induction by factors involved in inflammation gives a clear indication that *gro*/MGSA may exert a role in an autocrine fashion in the process of inflammation and early wound repair.

Materials and methods

Materials

Fetal calf serum, trypsin, gentamicin, medium 199, DMEM, medium F-10 were purchased from GIBCO. Endothelial growth supplement, human recombinant IL-1-α, IL-1-β, EGF, human plasma fibronectin and insulin were from Collaborative Research. Bovine serum albumin (BSA), bacterial endotoxin lipopolysaccharide (LPS), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), heparin, protein A–Sepharose, cycloheximide, phenylmethylsulfonyl fluoride (PMSF), human thrombin (>3000 NIH U/mg), hirudin, diisopropyl-fluorophosphate (DFP) and transferrin were obtained from Sigma. [^{35}S]Methionine, [^{35}S]cysteine and ^{32}P-labeled nucleotides were from Amersham Radiochemicals. Recombinant tumor necrosis factor (TNF-α) and lymphotoxin (TNF-β) were purified to homogeneity at Genentech. MGSA, a gift from Dr A.Richmond (Emory University, Atlanta, GA), was purified from Hs294 melanoma cells and was free of detectable LPS.

Cell cultures

Human melanoma cell line Hs294 was from Dr A.Richmond and grown in serum-free F-10 medium as described (Richmond and Thomas, 1986). Human umbilical vein endothelial cells were obtained from American Type Culture Center and grown to confluence in tissue culture dishes precoated with fibronectin as described (Hoshi and McKeehan, 1984). These cells were used at passages 2 or 3. Endothelial cells were identified by their typical cobblestone morphology and by positive immunofluorescence staining for von Willebrand factor.

Treatment of endothelial cells

All experiments were performed in 60 mm tissue culture dishes within 24 h after endothelial cells reached confluence. Cell monolayers were washed three times with warmed PBS and reagents (IL-1, TNF, LPS, TPA, cycloheximide) were added to the washed monolayers in complete growth medium. Cells were harvested at the indicated times and total cytoplasmic RNA was prepared (Maniatis *et al.*, 1982). The yield of RNA was ~30 μg/dish. When thrombin or thrombin plus inhibitors (hirudin and DFP) were used, confluent endothelial cells were pre-incubated overnight in serum-free medium containing insulin (1 μg/ml), trransferrin (5 μg/ml) and BSA (0.5 mg/ml). Cells were then refed with fresh medium and treated with thrombin (3 U/ml) or with thrombin plus inhibitors as described (Awbrey *et al.*, 1979; Low and Cunningham, 1982).

Northern analysis

Total cytoplasmic RNA (10 μg/each sample) was denatured at 65°C in the presence of formamide and formaldehyde fractionated in 1.0% agarose/formaldehyde gels (Maniatis *et al.*, 1982). The gels were then blotted to nitrocellulose filters. the 700 bp *Eco*RI fragment of the MGSA cDNA (Richmond *et al.*, 1988) was used as a hybridization probe, labeled with [α^{32}P]dCTP (Feinberg and Vogelstein, 1983) to specific activities of ~3 × 10^9 c.p.m./μg. Hybridizations were performed under high stringency conditions (Derynck *et al.*, 1988). The filters were then washed in 0.1 × SSC at 60°C for 30 min.

gro/MGSA antibody production

An oligopeptide beginning with an amino-terminal cysteine residue followed by the *gro*/MGSA carboxyl-terminal sequence (IEKMLNSDKSN) was synthesized using the solid-phase method and conjugated through the terminal cysteine residue via thioester linkage to soybean trypsin inhibitor. Rabbits were immunized with 1 mg conjugate emulsified with complete Freund's adjuvant. At 4-week intervals additional injections of 200 μg of the immunogen in complete Freund's adjuvant were given by multisite s.c. injections. The serum was evaluated for reactivity with the peptide by ELISA 10–14 days after each booster injection. This antibody is capable of recognizing a recombinant MGSA produced by mammalian cells or in a coupled *in vitro* transcription–translation system (unpublished data).

Metabolic labeling of endothelial cells and immunoprecipitation
Growth and treatment of cells were as described above, except that the reagents (IL-1, TNF, LPS, TPA and thrombin) were added to the washed cell monolayers in 1.5 ml of methionine- and cysteine-free medium. After 1 h, [^{35}S]methionine (150 μCi), [^{35}S]cysteine (100 μCi) were added and cells were incubated at 37°C overnight. The medium was collected, made 1 mM in PMSF, and clarified of cell debris in a microfuge. Samples were pretreated with 10 μl of rabbit normal serum, 20 μl of an undiluted suspension of protein A–Sepharose at 4°C for 1 h. The protein A–Sepharose was removed by centrifugation and the pretreated samples were reacted with the rabbit-anti-peptide antiserum at a 1:100 dilution and with 50 μl of protein A–Sepharose at 4°C overnight. The protein A–Sepharose beads were pelleted and washed three times in 0.1% Triton X-100, 0.02% SDS, 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 10 units of trasylol per ml. The washed beads were heated with 100 μl of SDS gel electrophoresis loading buffer at 100°C for 3 min. The beads were removed by centrifugation and the supernatant fluid analyzed by tricine–SDS–PAGE (Schagger and von Jagow, 1987).

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